CHARACTERIZATION OF BARLEY (*Hordeum vulgare* L.) WITH ALTERED CARBOHYDRATE COMPOSITION

A Thesis Submitted to the College of

Graduate Studies and Research

in Partial Fulfillment of the Requirements

for the Degree of Doctor of Philosophy

in the Department of Plant Sciences

University of Saskatchewan

Saskatoon

By

Eric Kwabena Asare

PERMISSION TO USE

In presenting this thesis in partial fulfillment of the requirements of a Postgraduate degree from the University of Saskatchewan, I agree that the Libraries of this university may make it freely available for inspection. I further agree that permission for copying of this thesis in any manner, in whole or in part, for scholarly purposes may be granted by the professor or professors who supervised this thesis work or, in their absence, by the Head of the Department or the Dean of the College in which this thesis work was done. It is understood that any copying or publication or use of this thesis or part thereof for financial gain shall not be allowed without my written permission. It is also understood that due recognition shall be given to me and to the University of Saskatchewan in any scholarly use which may be made of any material in my thesis.

Request for permission to copy or to make other use of material in this thesis in whole or part should be addressed to:

Head of the Department of Plant Sciences
51 Campus Drive,
University of Saskatchewan,
Saskatoon, Saskatchewan, Canada
S7N 5A8

ABSTRACT

The concentrations of storage constituents in barley (*Hordeum vulgare* L.) grain impact its use for malt, food, feed, and fuel. Characterization of major grain constituents such as carbohydrates (total dietary fiber, beta-glucan, starch and its components amylose and amylopectin, starch granule size and physical properties), protein and fat and their interaction during starch enzymatic hydrolysis will facilitate diversification of barley grain utilization for improved malt, food or feed applications. Improved understanding of genes and their structure associated with beneficial grain constituents will help in barley improvement program to develop novel cultivars with desirable grain constituents. Nine barley genotypes with varying amount of amylose in grain starch were used to: (i) determine grain carbohydrate (dietary fiber, beta-glucan and starch), protein and fat concentrations, and starch composition and structure on its enzymatic hydrolysis; (ii) characterize allelic variation in granule bound starch synthase 1 (*Gbss1*) and starch branching enzyme 2b (*Sbe2b*) to understand the molecular basis for variation in grain starch amylose concentration. Nine barley genotypes included, one normal (~25% amylose), three near waxy (< 5% amylose), two waxy (undetected amylose) and three increased amylose (> 38%) starch. Total starch concentration showed significant positive correlation with thousand grain weight (TGW) and negative correlation with amylose, total dietary fiber and protein concentrations. Starch granule sizes varied with percent amylose where the increased amylose genotypes produced significantly lower volume percentage of C-type granules (< 5 µm diameter) but significantly higher medium sized B granules (5-15 µm diameter). Amylopectin chain length distribution (CLD) was categorized into F-I type chains (6-11 dp); F-II (12-18 dp); F-III (19-36 dp) and F-IV chains (37-45 dp). Rate of starch hydrolysis was high in pure starch samples as compared to meal samples. Enzymatic hydrolysis rate both in meal and pure starch samples followed the order waxy > normal > increased amylose. Rapidly digestible starch (RDS) increased with a decrease in amylose concentration. Atomic force microscopy (AFM) analysis revealed higher polydispersity index of amylose in CDC McGwire and increased amylose genotypes which could contribute to their reduced enzymatic hydrolysis, compared to waxy starch genotypes. Increased β-glucan and dietary fiber concentration also reduced enzymatic hydrolysis of meal samples. Average linkage cluster analysis dendrogram revealed that variation in amylose concentration significantly ($p < 0.01$) influenced resistant starch concentration in

meal and pure starch samples. RS is also associated with B-type granules (5-15 µm) and amylopectin FIII (19-36 DP) fraction. *Gbss1* nucleotide sequences revealed considerable heterogeneity, with three genotypes with severely reduced GBSSI proteins and low amylose concentration had a 403 bp deletion in the promoter region. One previously described amino acid substitution D287V in CDC Alamo was confirmed and two new amino acid substitutions, G513W in CDC Fibar and Q312H in near waxy genotype SB94912 were identified as the likely causes of inactive GBSSI resulting in no amylose in starch granules. In the increased amylose genotype SB94983 A250T substitution was also observed, which can alter GBSS 1 enzyme specificity and could be a possible reason for increased amylose concentration. To facilitate studies of *GbssI* expression, positive assays for four allele variants were developed in this study. These markers may also be useful for monitoring introgression of respective *GbssI* alleles in barley improvement programs. The barley *Sbe2b* gene sequence analysis of the normal (CDC McGwire), near waxy (SB94912) and increased amylose (SH99250) genotypes, revealed 22 exons and 21 introns. The three *Sbe2b* alleles showed 21 polymorphic sites, present only in the introns, predicting that the processed SBE IIb transcripts to be identical in the three genotypes. The longest second intron of *Sbe2b*, which is known to regulate promoter activity in barley was identical between SH99250 and CDC McGwire, but SB94912 allele differed at 12 sites. The remaining nine polymorphic sites were present in introns 17, 18, 19 and 21. It is speculated that allelic variation in *Sbe 2b* second intron could be a reason for increased amylose concentration in SB94912. In summary reduction in amylose concentration can be due to changes in GBSS1 polypeptide, but the reasons for increased amylose starch are not yet clear. The DNA polymorphisms identified in this study can be used in a marker assisted selection strategy to follow the introgression of respective alleles in a barley improvement program.

ACKNOWLEDGEMENTS

I would like to sincerely thank my principal supervisor, Dr. Ravindra Chibbar (Research Chair in Grain Quality, Department of Plant Sciences) for accepting me from Ghana as his student, his kind support in financial terms and guidance for my Ph.D program. His insistence of quality work, precision and time consciousness has shaped my life and will help me in my future endeavors.

I wish to thank Dr. Monica Baga (Grain Quality laboratory, Department of Plant Sciences) for helping me in my research and also as a committee member. My sincere thanks go to other advisory committee members: Dr. Bruce Coulman (Head, Department of Plant Sciences), Dr. Brian Rossnagel (Barley and Oats Breeder, Crop Development Centre) and Dr. Murray Drew (Animal Feed Specialist, Department of Animal and Poultry Science) for their constructive comments and suggestions.

The financial assistance provided by Canada Research Chairs program, NSERC and the Department of Plant Sciences is very much appreciated. I also want to thank Dr. Pooba Ganeshan and other colleagues in the Molecular Crop Quality group for their help during my Ph.D studies. I am also grateful to the Biotechnology and Nuclear Agricultural Research Institute (BNARI) of the Ghana Atomic Energy Commission for releasing me for the Ph.D program. My sincere gratitude goes to my wife Juliana Asare and family members (Portia, Daniel, Angela and Eugene) for their support. My final thanks go to the Almighty God for sustaining my life and health to date.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ae	:	amylose extender
AFM	:	Atomic Force Microscopy
AGPase	:	ADP- glucose pyrophophorylase
ANOVA	:	Analysis of variance
ATP	:	Adenosine triphosphate
BG	:	Beta Glucan
CV	:	Coefficient of variation
CVD	:	Cardiovascular disease
CLD	:	Chain length distribution
CHD	:	Coronary heart disease
DBE	:	Debranching enzyme
DF	:	Dietary Fiber
DMSO	:	Dimethyl sulphoxide
DP	:	Degree of polymerisation
FAO	:	Food and Agricultural Organization
FIA	:	Flow injection analysis
3-PGA	:	3- phosphoglyceric acid
GBSS	:	Granule bound starch synthase
Glc-1-P	:	Glucose-1-phosphate
GI	:	Glycemic index
GOPOD	:	Glucose oxidase/peroxidase
GWD	:	Glucan water dikinase
HPLC	:	High performance liquid chromatography
LDL	:	Low-density lipoprotein
LMWC	:	Low Molecular Weight Carbohydrates
MMT	:	Million Metric Tons
MOPS	:	3-(N-morpholino) propanesulfonic acid
Pho	:	Phosphorylase
PUL	:	Pullulanase
RDS	:	Rapidly digestible starch
RFO	:	Raffinose Family of Oligosaccharides
RS	:	Resistant starch
RNAi	:	RNA interference
SBE	:	Starch branching enzyme
SDS-PAGE	:	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SDS	:	Slowly digestible starch
SS	:	Starch synthase
SEC–HPAEC	:	Size-exclusion chromatography- High performance anion-exchange chromatography
SGP	:	Starch granule protein

LIST OF APPENDICES

CHAPTER 1

INTRODUCTION

1.1 Barley (*Hordeum vulgare* L.)

Barley (*Hordeum vulgare L.*) is an annual herbaceous plant with fibrous root system. Barley is a member of the grass family *Poaceae* and descended from wild barley (*Hordeum spontaneum* K). Both forms are diploid with seven pairs of chromosomes (2n = 14). Barley is the major cereal grain used for malt production for beer and whiskey, and animal feed. Recently, barley is also being promoted for food and industrial purposes. Globally, barley is the fourth most important cereal crop after maize, rice and wheat in terms of quantity produced, area of cultivation (560,000 km^2) and consumption (The FAO Statistical Yearbooks 2008/2009). Barley is widely adaptable and is currently a major crop of the temperate areas where it is grown as a summer crop and in the tropics as a winter crop. However, barley likes to grow under cool conditions but it is not particularly winter hardy. Cultivated barley can be distinguished in to two types: two-row and six-row barley which may either be hulled or hulless (Bhatty, 1993; Powell et al., 1990). Canada is world's third largest commercial producers of barley accounting for 9% of total world production (FAO STAT 2009). In Canada, barley is the second most important cereal crop after wheat in terms of production, export and consumption. Alberta and Saskatchewan account for 45% and 43% respectively of total barley production in Canada (Statistics Canada, 2008).

Barley utilization is dependent on the relative concentrations of main storage components of a kernel. Carbohydrates (starch and non-starch), protein and lipids are the main storage constituents in a barley kernel. The other minor components include vitamins, minerals and phytochemicals. Starch is the major storage carbohydrate of barley ranging from 58-70% depending upon genotype, cultural practices and environmental conditions. The two main glucan polymers of starch are amylose (one-quarter) and amylopectin (three-quarters) organized into semi-crystalline water insoluble granules. Amylose is predominantly linear with sparse branches while amylopectin is highly branched glucan polymer. The amylose to amylopectin ratio, amylopectin degree of polymerization (~10^5) and average chain length affect physicochemical properties of starch and influence its end-use. Barley like others in the *Poaceae* family has two

types of starch granules, large A-type (10-35 μm) and small B-type (1-10 μm), which also influence starch physico-chemical properties and end-use (Lindeboom et al., 2004). Starch granule size, composition and fine structure also influence starch enzymatic digestibility, which has attracted considerable attention for the benefit of human health and disease prevention. Based on digestibility, starch is classified as rapidly digestible starch (RDS), slowly digestible starch (SDS) and resistant starch (RS). RDS and SDS is a portion of starch digested within 30 min and 120 min respectively, upon ingestion, while RS remains undigested in the small intestine and is broken down by the large intestine micro-flora. RS is positively correlated with amylose concentration. Foods containing RS have several human health benefits such as low glycemic index, promotion of gut health and reduction of cardiovascular diseases (Chibbar et al., 2010). However, for animal feed increased amylose starch is not desirable, because it forms stable complexes with lipids and resists α-amylase degradation and reduces feed efficiency (Kwaśniewska-Karolak et al., 2008). Besides starch, barley kernel contains simple sugars such as glucose, fructose and maltose with concentrations ranging from 0.1 to 0.2% (Holtekjølen et al., 2008).

Beta-glucan and total dietary fiber are the most important form of barley non-starch carbohydrates because of their effect on malting and health implications. High beta-glucan and dietary fiber are desired in foods as they lower blood cholesterol and reduce risk of cardiovascular disease in humans (Izydorczyk and Dexter, 2008). However, increased beta-glucan concentration negatively effects malting because it lowers malt extract values, increases filtration difficulty and produces precipitates in beer (Matthies et al., 2009). Protein concentration in Canadian barley genotypes varies from 10 to 15% however the range is wider (7 to 25%) in other improved genotypes (Newman and Newman 2005) depending on end use. The concentration of lipids in barley endosperm (mostly in the embryo) ranges from 2.0 to 7.3% depending genotype and extraction methods (Morrison 1993a; Qian et al., 2009).

Several approaches such as agronomic, breeding and molecular techniques have been used for improvement of barley grain components. New avenues for altering amylose: amylopectin ratio in starch is available based on knowledge of key enzymes involved in starch biosynthesis. In barley (Regina et al., 2010), wheat (Regina et al., 2006), durum (Lafiandra et al., 2009) reported altered amylose concentration and changed starch physicochemical properties by RNA interference (RNAi) mediated inhibition of SBEIIa and / or SBE IIb. Apparent increased

amylose concentration in maize (Boyer and Preiss 1981) and rice (Mizuno et al., 1993) and potato (Schwall et al., 2000) were associated with mutations in starch branching enzyme genes. Barley *sex6* mutant (Morell et al., 2003) and the cultivar 'Himalaya 292' (Bird et al., 2004) have lesions in starch synthase II and starch synthase IIa, respectively, resulting in increased amylose grain starch. Allelic differences in wheat lines denoted as SGP-A1, SGP-D1 and SGP-B1 (Yamamori et al., 2000) were correlated with increased amylose starch, change in amylopectin structure and physical properties. In this study, three barley genotypes with increased amylose concentration were compared to five reduced amylose starch (waxy or near starch) genotypes to understand the relationship between change in starch composition and structure to other grain constituents and their influence on *in vitro* enzymatic hydrolysis of starch. Another part of this study was to understand the molecular basis of variation in starch composition and structure in selected barley genotypes.

1.2 Hypothesis

a) Alteration in starch composition and amylopectin structure change *in vitro* enzymatic digestion of starch.

b) Granule bound starch synthase 1 (GBSS1) and starch branching enzyme (SBE) cause changes in starch composition and amylopectin structure.

1.3 Objectives

The objectives of the study were:

a) Analyze grain storage constituents of barley genotypes with altered carbohydrate composition.

b) Elucidate starch granule and amylopectin structure and their relationship to *in vitro* enzymatic starch hydrolysis.

c) Characterize genes for two major starch biosynthetic enzymes (GBSSI and SBE) implicated in amylose biosynthesis in increased amylose starch barley genotypes.

CHAPTER 2

LITERATURE REVIEW

2.1 Major crops in Canada

Cereal and oilseed crops are the dominant crops of the Canadian agriculture, followed by forages, potatoes, pulses and fruits. The major oilseed crops include canola, soybeans and flax, whereas wheat, barley, corn, oats and rye are the most common cereals. During the 2007 to 2009 time period, wheat, barley, canola, soybean and oats contributed 45%, 24%, 10%, 8%, and 5% respectively to the Canadian agricultural production (Statistics Canada, 2009). Since the last 75 years, cereal production on the Canadian Prairies occupies 75-80% of the cultivated acreage and is dominated by wheat, barley and oats. Barley is mainly destined for animal feed or production of malt for domestic or export markets. Canadian barley constitutes 8.1% of global trade and is valued at $1.9 billion per year (FAOSTAT, 2009).

2.2 Barley Taxonomy

Barley (*Hordeum vulgare* L.*)* is a member of the *triticeae* tribe of grass family *Poaceae* (*Gramineae)*. All the *Poaceae* members have likely evolved from a common ancestor (Devos, 2005), thus the Poaceae family can be considered monophyletic. The *Triticeae* tribe consists of 350 to 500 species (Bothmer et al., 1995), among which several important cereal and forage crops such as wheat (*Triticum spp.*), rye (*Secale cereale* L.), and crested wheatgrass (*Agropyron cristatum*) are included. However, the taxonomic delimitation of the tribe has not been fully resolved (Barkworth, 1992).

Hordeum comprises of a group of well defined and easily recognized plants made up of 32 species and 45 taxa with the basic chromosome number of seven (Bothmer et al., 1995). Most of the *Hordeum* members are diploid (2n = 2x = 14), tetraploid (2n = 4x = 28) or hexaploid (2n = 6x = 42) (Bothmer et al., 1995; Bothmer et al., 2003) (Table 2.1). Two species, *H. bulbosum* L. and *H. brevisubulatum* L. are autoploids and a few aneuploid *Hordeum species* have evolved due to elimination or duplication of chromosomes (Linde-Laursen et al., 1986b). The two-row shattering (*H. spontaneum* K.) and non-shattering (*H. distichum* L) barley and the six-row shattering (*Hordeum vulgare* L.) and non-shattering (*H. agriocrithon* E. Åberg) barley were previously considered as four separate species. Today, they are all known as *Hordeum vulgare* L.

Table 2.1. Taxa in the genus *Hordeum*, their distribution, chromosome numbers and life forms

Species	Subspecies	2n	Life forms*	Distribution**
H. vulgare L	2	14	A	Cultivated and E. Mediterranean
H. bulbosum L.		14, 28	P	Mediterranean
H. murinum L	3	14, 28, 42	A	Europe, Mediterranean to Afghanistan
H. pusillum Nutt		14	A	USA, N Mexico and S Canada
H. intercedends Nevski		14	A	SW California and N Mexico
H. euclaston Steud.		14	A	C. Argentina, Uruguay and S Brazil
H. flexuosum Steud		14	A/P	Argentina and Uruguay
H. muticum Presl		14	P	W South America
H. chilense Roem & Schult.		14	P	C. Chile and W Argentina
H. cordobense Bothm.		14	P	Argentina
H. stenostachys Godr		14	P	Argentina, Uruguay and S Brazil
H. pubiflorum Hook	2	14	P	W. Argentina, Chile, Bolivia, Per
H. comosum Presl		14	P	Chile and W Argentina
H. jubatum L.		28	P	W North America to E Russia
H. arizonicum Covas		42	A/P	S. USA and N. Mexico
H. procerum Nevski		42	P	C. Argentina
H. lechleri (Steud) Schenk		42	P	Chile and Argentina
H. marinum Huds.	2	14, 28	A	Mediterranean to Afghanistan
H. secalinum Schreb		28	P	W. Europe and N. Africa
H. capense Thunb.		28	P	South Africa and Lesotho
H. bogdanii Wil.		14	P	C. Asia
H. roshevitzii Bowd.		14	P	S Siberia, Mongolia, N China
H. brevisublatum (Trin.) Link	5	14, 28, 42	P	Asia
H. brachyantherum Nevski	2	14, 28, 42	P	W N America to Kamchatka
H. depressum (Scribn. & Sm) Rydb		28	A	W. USA
H. guatemalense Bothm et al.,		28	P	N. Guatemala
H. erectifolium Bothm et al.,		14	P	C. Argentina
H. tetraploidum Covas		28	P	S. Argentina
H. fuegianum Bothm et al.,		28	P	S. Argentina S Chile
H. patodii Covas		42	P	S. Argentina S Chile
H. patagonicum (Haum.) Covas		14	P	S. Argentina S Chile

*A= annual; P= perennial; A/P= annual or weakly perennial; Adapted from Bothmer et al., 2003

after the discoveries that the differences in shattering and spike types are due to two complementary brittle rachis (*Btr*) genes (Zohary and Hopf 2000). The shattering character of *H. spontaneum* is caused by brittle rachis which enables seed dispersal in the wild. Another form of shattering occurs when breaking of rachis leads to complete loss of spike (Kandemir et al., 2000). A mutation in one or both of the tightly linked *Btr1* and *Btr2* on chromosome 3H resulted in development of shattering-resistant barley (Azhaguvel et al., 2006). The cultivated form of barley (*Hordeum vulgare* L.) also includes a group of barley lines derived from crosses between two-row and six-row barley that were previously denoted as *H. intermedium* (Jui et al., 1997). Cultivated barley is quite similar to its wild progenitor *H. spontaneum*, with the exception of having broader leaves, tougher ear rachis, shorter and thicker spike, larger grains and shorter stems and awns.

Hordeum genus is widespread in temperate areas and occurs in several biotopes worldwide. Most native *Hordeum* species have their diversity centers in southern South America, western North America, the Mediterranean, and Central Asia (Bothmer et al., 2003). The global barley inventory held by centres such as the International Center for Agricultural Research in the Dry Areas, Aleppo, Syria includes nearly 12,500 accessions of *H. vulgare* subsp. *spontaneum* originating from 25 countries concentrated to the western part of the Fertile Crescent (Jilal et al., 2008).

2.3 Barley classification

Barley is classified by the number of kernel rows in a spike. Two forms are cultivated; two-row and six-row barley. The number of rows on the ear is controlled by two alleles at the *Vrs1* locus on chromosome 2H (Powell et al., 1990). Only one spikelet per node is fertile in two-row barley (*vrs1*, *vrs1*), whereas all three spikelets produce seeds in six-row forms (*Vrs1*, *Vrs1*) (Fig 2.1 a, b). The grains of two-row are more circular and have a higher test and grain weight than six-row kernels (Tanno and Takeda 2004). In addition, two-row barley produces grain with higher starch and protein concentrations (Marquez-Cedillo et al., 2001), and thus, the two-row grain has a higher feed conversion (> 4.6%) than six-row grain (Fox et al., 2009). Two-row genotypes also have the advantage of being more resistant to lodging than six-row types (Berry et al., 2006). The higher number of kernels per spike developed in six-row varieties translated to

Figure 2.1 Barley spike and seed types
(a) two-row, (b) six-row, (c) hulled and (d) hulless (**www.barleyworld.org**)

a 20 to 27% higher yield than two-row types. The fiber and beta-glucan content in six-row grain is relatively high, which is of advantage for production of fiber-rich barley food products. Another barley classification is based on the kernel being hulled or naked (Fig 2.1 c, d). The

naked trait is controlled by a single recessive gene on chromosome arm 7HL (Pourkheirandish and Komatsuda 2007). Wild barley and some of the cultivated forms are hulled with paleas fused with kernel after threshing. Paleas of hulless or "naked" barley (*Hordeum vulgare* L.) are easily removed by threshing. The naked barley is mostly used for food and feed whereas the hulled barley is preferred for malting.

2.4 Origin and domestication

Archeological excavations have identified barley in many pre-agricultural and incipient sites in the Near East, Southeast Asia and Ethiopia where it was one of the first crops cultivated for more than 10,000 years ago (Zohary and Hopf 2000). The Fertile Crescent which stretches from Israel and Jordan to south Turkey, Iraq, Kurdistan, and south-western Iran still remains the centre of origin and is the primary habitat for barley's wild progenitor, *H. spontaneum* (Harlan and Zohary 1966). The Mediterranean marquis, abandoned fields, and roadsides are considered as secondary habitats for *H. spontaneum*. *H. spontaneum* is also present in the marginal habitats in the Aegean region, south-eastern Iran, and central Asia, including Afghanistan and the Himalayan region (Zohary and Hopf 2000). The discovery of *H. spontaneum* in several locations suggests that barley (*H. vulgare*) is an oligocentric crop based on its evolutionary pattern (Åberg, 1938; Bekele, 1983; Molina-Cano et al., 1987). This hypothesis is supported by a study of 317 wild and 57 cultivated barley which indicated that barley was brought into cultivation in the Israel-Jordan area and that the Himalayas is another region of domesticated barley diversification (Badr et al., 2000).

2.5 Barley distribution

Barley is cultivated over a wide range of environments from 70^{o}N in Norway to 46^{o}S in Chile. As barley is a relatively drought tolerant crop, it can be grown in many countries with dry climate such as Afghanistan, Pakistan, Eritrea and Yemen and areas in Northern Africa and Western Asia. Barley is also widely cultivated on high altitude mountain slopes in Tibet, Ethiopia and the Andes. Europe has the largest area under barley cultivation (ca. 27.3 million hectares) followed by Asia (ca. 12.2 million hectares), North and South Americas (ca. 6.6 million hectares), Africa (ca. 4.9 million hectares) and the Oceania (ca. 4.5 million hectares) (FAOSTAT, 2009). The Russian Federation is the leading barley producer (12%), followed by Spain (9%), Canada (8%) and Germany (8%) and France (7%) (FAOSTAT, 2009).

2.6 Barley growth

Barley is a C3 plant and prefers climates with cool temperatures (15-30 ^{o}C) and an annual precipitation of 500-1,000 mm. Adaptation to soil salinity and alkalinity is good, but barley is less tolerant to acidic and wet soils (Bhatty, 1999). Barley grows rapidly, out-competes many weeds and reaches maturity earlier than wheat and oats. Most of the wild barley have winter growth habit (Karsai et al., 2005), which demands a period of low temperatures (around 10 ^{o}C) to become flowering competent. Cultivated barley with winter habit is grown in tropical areas, whereas spring types dominate in temperate regions. The winter hardiness of Canadian winter barley is lower than that of Canadian winter wheat (*Triticum aestivum* L.), rye (*Secale cereal* L.) and Triticale (× *Triticosecale* Wittm. ex A. Camus), and is only grown on a small scale in southern Ontario.

2.7 Uses of barley

The most important uses of barley are in the malting and brewing industry for beer and whiskey production, animal feed and human food. In Canada, about 83% of produced barley is used as livestock feed whereas 12% and 5% are destined for malting and other purposes, respectively. There has been a higher demand for food and malting barley in the last five to ten years as a result of increased health awareness and favorable market prices (Baik and Ullrich 2008). Novel uses of high β-glucan barley in the nutraceutical industry has emerged lately (Delaney et al., 2003) and industrial applications of high-amylose barley starches are under investigation to diversify barley utilization (Ganeshan et al., 2008).

2.7.1 Animal feed

Barley is often used for animal feed although the nutritive value is lower than corn or wheat. Both two- and six-row hulless barleys are grown to produce animal feed. In addition, a considerable amount of hulled malting barley with inadequate malting quality enters the animal feed market. This makes feed barley a non-homogeneous commodity with varying nutritive value. The two-row and six-row hulless barleys produced in Canada for feed are relatively high in protein (14-15%), but the two-row grain is preferred as it has a comparatively higher carbohydrate (starch) content (Fregeau-Reid et al., 2001) and is more digestible by monogastric farm animals such as poultry and swine.

To aid digestion of the fibrous hulls of hulled barley, the rations of monogastric animals are often supplemented with beta-glucanase (Mathlouthi et al., 2003).

Besides hulls, phytate is another seed component that negatively affects barley utilization as animal feed. Phytic acid efficiently chelates multivalent cations such as zinc, calcium, copper, iron, magnesium, and aluminium making the minerals unavailable for absorption (Adams et al., 2002). Among the minerals, zinc is the most susceptible to phytate complexation. A reduction of phytate production in barley cultivar HB379 (Roslinsky et al., 2007) has doubled the availability of phosphorous and zinc for broilers (Linares et al., 2007). Although barley is popular as animal feed, grain with a high starch concentration is not suitable for ruminants. A rapid starch fermentation in rumen results in a pH drop, which reduces fiber digestion and causes digestive disorders. Depression in milk fat content is seen when high-starch barley is used as feed for lactating cows (Larsen et al., 2009). It is therefore important to consider seed composition when selecting barley grain for ruminants and non-ruminants, respectively.

2.7.2 Malting and brewing

About 10% of barley produced worldwide is used to make malt for brewing beer. The malting cultivars include hulled, hulless, two-row and six-row varieties, but the hulled barley is preferred as hulls contributes to flavor and aids filtering during the brewing process (Gunkel et al., 2002). Malting barley varieties are generally developed for a specific market e.g. domestic brewing or for export. Three-quarters of the area seeded to barley in Canada is occupied by two-row malting barley, whereas barley growers in the US prefer six-row white aleurone varieties. The six-row malting barley produced in Canada contributes 5% to the global barley trade and is mainly exported to the US market (Statistics Canada, 2009).

The physical, chemical and biochemical properties of barley grain can have a large impact on the malting process and quality of beer. Kernel physical characteristics such as germination percentage, germ growth, kernel maturity, size, amount of seed-borne diseases and frost damage are factors that affect malting. The amount of grain starch, protein, β-glucan and their interactions during grain filling affect grain hardiness with effects on the yield of malt extract (Psota et al., 2007). The alpha amylase level is another factor that determines the amount of malt extract. Preferred malting barley varieties are generally soft (Gupta et al., 2010) with protein levels ranging from 10.5% to 13.0% for six-row types and 10.5% to 12.5% for two-row

varieties (Dusabenyagasani, 2003). Barley with high protein concentration (> 15%) is not used for malting as it requires a long steeping time, has erratic germination and produces low malt extract (Swanston and Molina-Cano 2001). Discolored barley grain is also unsuitable for malting due to undesirable flavors produced in beer by the breakdown of phenolics (Mussatto et al., 2006). A successful sustenance of malting barley export market demands proper selection of cultivars with appropriate malting characteristics.

2.7.3 Nutrition and human health

About 2% of the global barley production is used for food (Baik and Ullrich 2008). The preferred barley for food use is clean, thin-hulled, bright yellow-white, plump, medium-hard and uniform in size. A few two-row and six-row hulless genotypes with a minimal cleaning requirement meet these specifications. Barley is nutritionally rich because it is has a high carbohydrate concentration, moderate protein concentration, high dietary fiber content and is a good source of selenium, phosphorus, copper and manganese (Ames et al., 2006). Dehulled, polished and milled barley is often used in porridge and soups, and as a substitute for rice in certain Asian countries e.g. Iran. Also a substantial amount of barley is used in baked foods such as breads, grits, noodle and pilaf in India and surrounding countries.

Barley-based foods provide several positive effects on the human digestive system. Consumption of barley increases bulk and reduces transit time of fecal matter, which is associated with a lower frequency of hemorrhoids and colon cancer (Tsai et al., 2004). Fermentation of barley's insoluble dietary fiber in large intestine produces short-chain fatty acids such as butyric acid that help to maintain a healthy colon (Behall et al., 2004). Other fermentation products such as propionic and acetic acids provide fuel for liver and muscle cells (Liu, 2004). Propionic acid is also known to inhibit HMG-CoA reductase involved in cholesterol biosynthesis in liver (Erkkila et al., 2005), thus lowering blood cholesterol levels.

One of the important dietary fibers produced by barley is the soluble glucan polymer β-glucan (1→3, 1→4)-β-D-glucan). The concentration of β-glucan in barley kernels is normally < 5% (Izydorczyk et al., 2000), but genotypes producing waxy or high-amylose starch generally have a higher concentration of β-glucan and dietary fiber (Izydorczyk et al., 2000). The presence of β-glucan in diets increases the viscosity of foods during digestion leading to lower glucose absorption and reduced blood glucose level measured as glycemic index (GI) (Jenkins et al.,

1981). Foods with a low GI is preferred to decrease the risk of diabetes in humans. Beta-glucan also has a positive effect on cholesterol levels, as the fiber absorbs and removes bile acids produced from cholesterol in the liver. The absorption of bile acids triggers the liver to produce more bile acids from cholesterol (Brennan, 2005) and the net effect is a reduction in blood cholesterol levels (Behall, 2004). Barley fiber is also a good source of niacin, a B-vitamin that reduces platelet aggregations that cause blood clots and lowers the levels of total cholesterol, lipoprotein and free radicals which oxidize low-density lipoprotein cholesterol. Thus, niacin protects against cardiovascular diseases (Jood and Kalra, 2001). Consumption of food with 21 g fiber per day have been suggested to lower the chances of coronary heart and cardiovascular diseases by 12-15% and 10-11%, respectively (Jensen et al., 2004).

As various health claims are associated with barley grain consumption, future barley based food products are aimed at regulation of blood sugar levels in diabetics, reducing cholesterol and lowering the incidence of heart disease. Besides low GI foods being desirable for diabetics, they are also beneficial for athletes requiring a slow release of glucose into the blood.

2.8 Barley kernel composition

The three main components of the barley kernel are: endosperm, bran and germ (Fig 2.2). About 83% of the kernel weight constitutes the endosperm, which is mainly composed of carbohydrates (70-77%) and protein (12-16%) together with traces of vitamins and minerals (< 2%). The bran makes up to about 14% of the kernel weight and is composed of small amount of protein (< 3%), trace minerals (3-5%), and relatively large quantity of three major B vitamins (3 - 6%) and non-starch carbohydrates providing dietary fiber (4.5-15%). About 2.5% of kernel weight constitutes the germ, which includes the embryo. The germ contains mostly high quality protein (12-20%), lipids (1.5-5%) and is a good source of B-complex vitamins and trace minerals (< 2.5%) (USDA Nutrient Database).

2.8.1 Carbohydrates

Carbohydrates are organic molecules with the general formula $C_x(H_2O)_y$. Simple sugars like glucose, fructose and galactose make up the building blocks of carbohydrates, which can be classified as monosaccharides, disaccharides, oligosaccharides or polysaccharides (Chibbar et al., 2004). Carbohydrates are the main energy source for humans, and can from a nutritional aspect be divided into available carbohydrates (readily digested in the small intestine) and

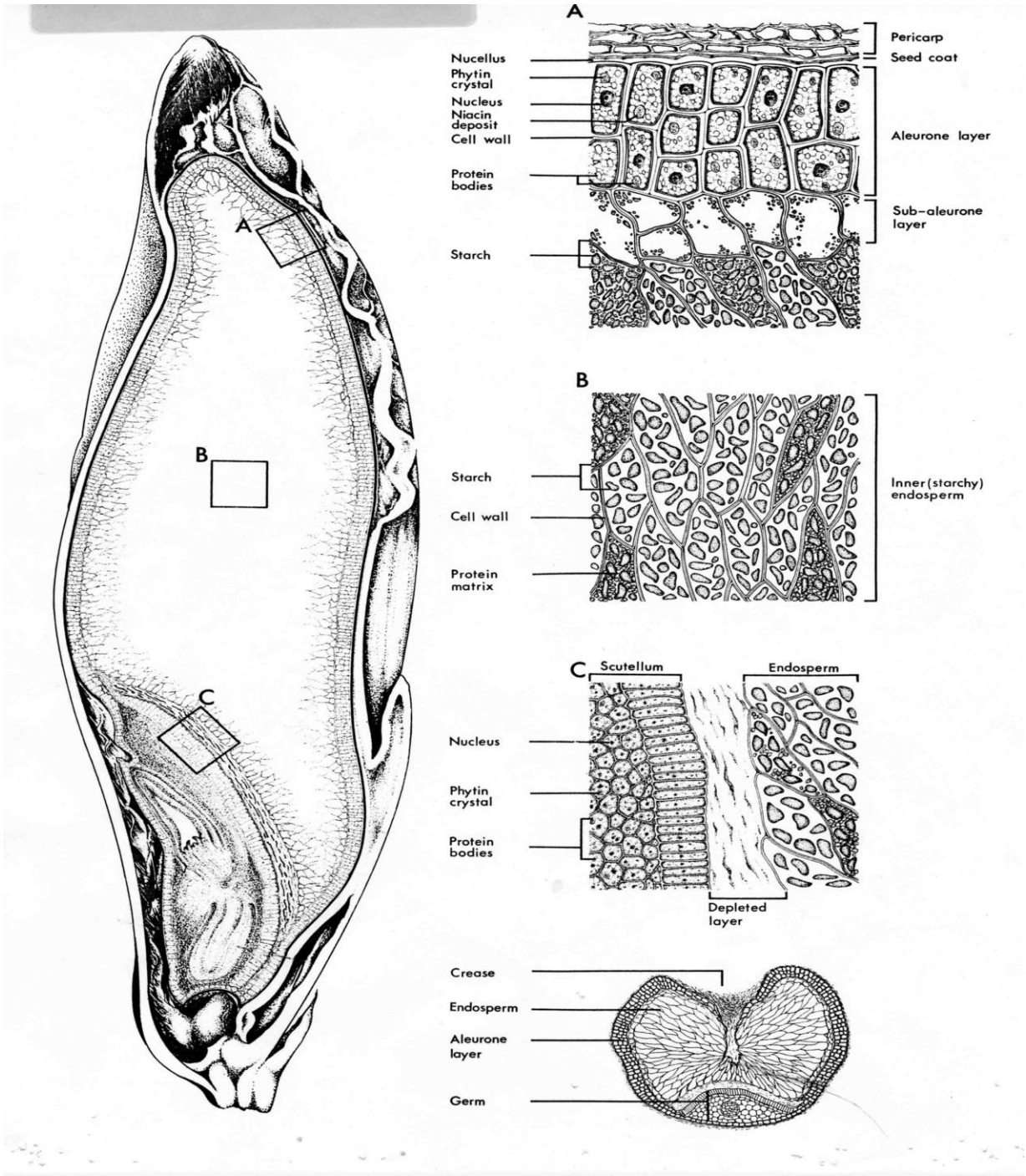


Figure 2.2 Barley kernel showing different components and composition (Adapted with modification from www.cwb.ca/).

unavailable carbohydrates (not digested in the small intestine but fermented in the large intestine). Glucose and fructose are examples of available carbohydrates, whereas many oligosaccharides, resistant starches, pectins, β-glucan, cellulosic and / non-cellulosic polysaccharides and hemicelluloses are regarded as unavailable (Chibbar et al., 2004).

2.8.1.1 Monosaccharides

The mature endosperm of normal barley contains 2-3% monosaccharides, mostly in the form of glucose and fructose (Table 2.2). Higher concentrations of simple sugars are found in hulless (2-4%), high lysine (2-6%) and high sugar (7-13%) barley grain (Holtekjølen, et al., 2008). Other monosaccharides such as fucose, arabinose, xylose, ribose, deoxyribose galactose and mannose are produced in grain to form oligo-and polysaccharides, glycosides, glyco-lipids or glyco-proteins (Holtekjølen, et al., 2008).

2.8.1.2 Disaccharides

The two most important disaccharides in barley grain are sucrose and maltose. The concentration of sucrose ranges from 0.74-0.84%, with close to 80% present in the embryo (MacGregor and Fincher 1993). Sucrose serves as an important precursor for starch biosynthesis, and can be accumulated to relatively high levels (7%) in *waxy* genotypes defective in amylose biosynthesis (Batra et al., 1982). Maltose accumulates to concentrations of 0.1-0.2% in barley endosperm as a result of starch amylolytic activities (Sopanen and Lauriere 1989). Due to increased starch-bound α-amylase activity in *waxy* genotypes, the maltose concentration can reach 0.4% (Nielsen et al., 2009).

2.8.1.3 Oligosaccharides

Oligosaccharides are polymers of 3-20 glucose units (Chibbar et al., 2004). Raffinose, myo-inositol, fructosans and bifurcose are some of the oligosaccharides present in barley kernels. The raffinose concentration is 0.3 to 0.8%, (Table 2.2) and 80% of it occurs in quiescent embryos (Andersen et al., 2005), where it has a role in seed desiccation and constitutes a carbon source at the early stages of seed germination (Sreenivasulu et al., 2008). Barley kernels also contain fructo-oligosaccharides (fructans) which contain up to ten fructosyl residues (Janthakahalli, 2004). Fructans enhance drought tolerance in barley (Janthakahalli, 2004) and higher oligosaccharides concentrations in barley kernels enhance seed survival under adverse

Table 2.2. Composition of hulled and hulless barley grain.

	Hulled		Hulless	
Compound	Mean (g/kg dry weight)	Range	Mean (g/kg dry weight)	Range
Starch	58.2	57.0 – 65.4	63.4	60.1 – 75.2
Protein[a]	13.7	8.2 – 15.4	14.1	12.1 – 16.6
Sugars	3.0	0.5 – 3.3	2.9	0.7 – 4.2
Lipids	2.2	1.9 – 2.4	3.1	2.7 – 3.9
Fiber	20.2	18.8 – 22.6	13.8	12.6 – 15.6
Ash	2.7	2.3 – 3.0	2.8	2.3 – 3.5

[a] Determined by the Kjeldahl method using N x 6.25 factor.
Source: MacGregor and Fincher 1993.

weather conditions (Bønsager et al., 2010).

2.8.1.4 Polysaccharides

Polysaccharides are glucose polymers synthesized by plants as storage carbohydrates (starch and β-glucan) or as structural carbohydrates (cellulose, chitin and arabinoxylans) (Chibbar et al., 2004).

2.9 Starch

Starch is the major carbohydrate in a barley kernel and the most extensively studied storage compound because of its direct effect on feed, malt, beer and food quality. The concentration of starch in barley kernels ranges from 45-65%, but in some hulless grain, the starch concentration reaches 75% (Table 2.2). Starch concentration in barley grain is inversely related to protein, non-starch carbohydrate and lipid concentrations (Newman and McGuire, 1985)

The two main glucan polymers of plant starches are amylose and amylopectin (Fig. 2.3 A, B; Table 2.3). Normal starch of barley kernels is made up of 22-26% amylose and 74-78% amylopectin. The amylose molecules consist of 100-10,000 D-glucose units linked by α-(1→4) bonds (Fig. 2.3 C; Table 2.3) and relatively few (> 0.5%) α-(1→6) linkages forming branches (Fig 2.3 D, Table 2.3). Thus, the degree of polymerization (dp) for amylose is 100-10,000 (Hung et al., 2008; Takeda et al., 1990).

Amylopectin is one of the largest molecules in nature with a molecular weight of 10^8 Daltons (Table 2.3). About 5% of the glucose linkages on amylopectin form branches. Each branch is about 20 to 30 glucose units in length, with a hydrodynamic radius of 21-75 nm making the amylopectin molecule bushy and nearly spherical in shape (Hizukuri, 1985). Due to frequent branching, viscosity of amylopectin in dilute solutions is lower than that of amylose. The amylopectin molecules are heterogeneous with average values of dp and chain lengths, where chain length is defined as the total number of glucan molecules divided by the number of non-reducing end groups (Manners, 1989). Three types of glucan chains can be distinguished in amylopectin molecules and they are denoted A-, B- and C-chains (Fig 2.3 B) (Manners, 1989). The frequent outer A-chains (dp < 12) are unbranched and linked to the inner branched B-chains (dp 13 to 35). The B-chains are attached to the center C-chain (dp < 36), which contains the only reducing group. Longer B-chains connect between clusters and shorter B-chains remain within the cluster (Bertoft et al., 2008). The numerous glucan ends on amylopectin are exposed for addition or removal of glucose units by enzymatic action.

The morphology and crystallinity of plant starches can be classified as A-, B- or C- type based on X-ray diffraction patterns (Hung et al., 2008). The A-type pattern is caused by amylopectin with short average lateral branch chains (Buleón et al., 1998) and is seen in starches of cereals like corn (Abd Allah et al., 2006), wheat (Hung et al., 2008), barley (Song and Jane 2000), and rice (Kubo et al., 2008). The B-type pattern is derived from amylopectin with long side chains containing distant branching points (Butrim et al., 2009) and is present in root and tuber starches of potato (Kim and Lee 2002), tapioca (Butrim et al., 2009) and taro (Hoover, 2001) . The C-type structure is a mixture of both A- and B- types (Wang et al., 2008). Legume starches such as pea contain both A and B type starch crystalline structure, with A-type structure in the periphery and B-type in the centre of starch granule (Gernat et al., 2006).

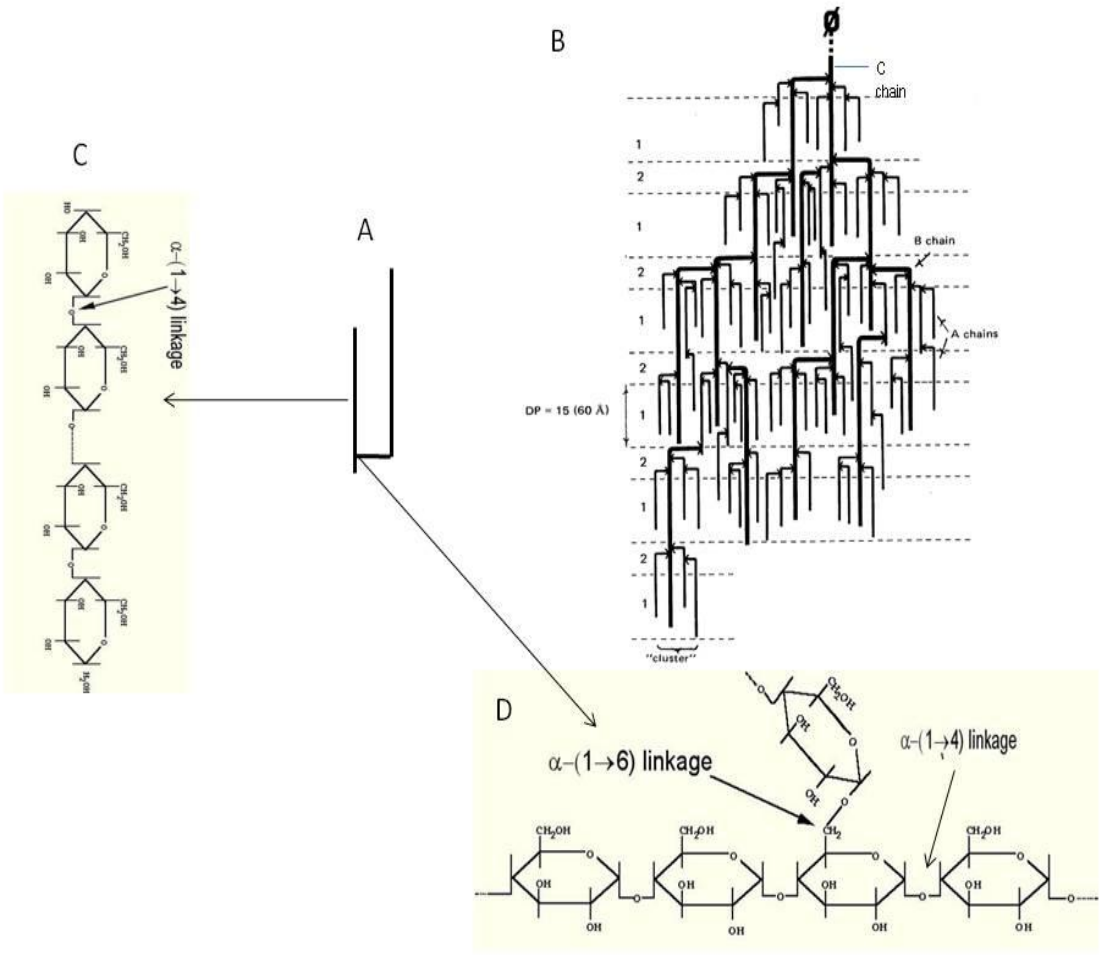


Figure 2.3 Schematic illustration of starch polymers.
Model structures of amylose (A), amylopectin (B), linear α-(1→4) glucan chain (C) and α-(1→6) branch point are shown. Reproduced from Robin et al., (1974) with modifications.

Starch susceptibility to enzymatic hydrolysis varies with botanical source, starch granule distribution and ratio between amylose and amylopectin (Srichuwong et al., 2005) (Table 2.2; Fig 2.4). Cereal starches hydrolyse readily as compared to starches from legume or tuber crops (Srichuwong et al., 2005). An increase in amylose concentrations to 35% slows down the hydrolysis rate of cereal starches, whereas elimination of amylose (*waxy* starch) increases the rate of hydrolysis (Srichuwong et al., 2005). Thus, it has been suggested that amylopectin fine structure which affects starch granule morphology affects the rate of enzymatic starch hydrolysis (Srichuwong et al., 2005).

Table 2.3 Properties of amylose and amylopectin

Property	Amylose	Amylopectin
General structure	essentially linear	heavily branched
Molecular weight	~10^6 Daltons	~10^8 Daltons
Color with iodine	dark blue	purple
λ_{max} of iodine complex	~650 nm	~540 nm
Iodine affinity	19-20%	< 1%
Average chain length*	100-10,000	20-30
Degree of polymerization*	100-10,000	3×10^5 - 3×10^6
Solubility in water	Variable	stable
Stability in aqueous solution	Retrogrades	stable
Conversion to maltose[a]	~70%	~55%

* glycosyl units
[a] conversion by crystalline β- amylase
Reproduced from: BeMiller and Whistler, (2009) and Suortti et al., 1998

2.9.1 Non starch polysaccharides (NSP)

Non-starch polysaccharides such as β-glucan, arabinoxylans and cellulose are abundant in the cell walls of hull, endosperm tissue and aleurone layer of barley kernels (Holtekjølen et al., 2006). They are all classified as total dietary fiber (TDF). The β-glucan concentration for most barley genotypes ranges from 3-5%, however some hulless, *waxy* and high amylose genotypes have concentrations in 8-10% range (Izydorczyk et al., 2000). Arabinoxylan concentration in barley kernel ranges from 3-5%. Cellulose is the primary component of barley hulls and the cellulose concentration in hulled and hulless barleys range from 4.1 to 4.8% and 2 to 2.9%, respectively (Holtekjølen et al., 2006). Depending on end-use of barley grain, non-starch polysaccharides can have positive or negative effects.

Table 2.4. Plants with uni-modal and bimodal starch granule size distribution.

Starch source	Diameter (μm)	Granule shape	Reference
Buckwheat	2-4	Polygonal	Zheng, 1997
Corn	2-30	irregular, polyhedric	Takeda et al., 1990
Waxy Corn	3-26	irregular, polyhedric	Takeda et al., 1990
Oats	2-14	small, irregular	Zheng, 1997
Rice	2-10	Polygonal	Zheng, 1997
Wild Rice	2-8	irregular, fused	Wang, 2002
Small Millet	0.8-10	Polygonal	Kumari, 1998
Potato	5-100	large oval	Hoover, 2001
Tapioca	4-35	smooth, irregular	Hoover, 2001
Taro	2-3	smooth, oval, round	Jane et al., 1992
Amaranth	1-2	uniformly sized	Jane et al., 1992
Quinoa	0.5-5	Oval	Zheng, 1997
Canary grass	1.5-3.5	smooth, small, oval	Abdel Aal et al., 1997
Barley	11-32 (A-type)	Discs	Tang et al., 2001
	1-10 (B-type)	Round	
Wheat	10-35 (A-type)	discs/ spheres	Evers 1973
	< 10 (B-type)	Round	
Rye	22-36 (A-type)	Discs	Jane et al., 1994
	2-3 (B-type)	Round	
Triticale	22-36 (A-type)	Discs	Jane et al., 1994
	< 5 (B-type)	Round	

Reproduced with modifications from Lindeboom et al., 2004; BeMiller and Whistler, 2009.

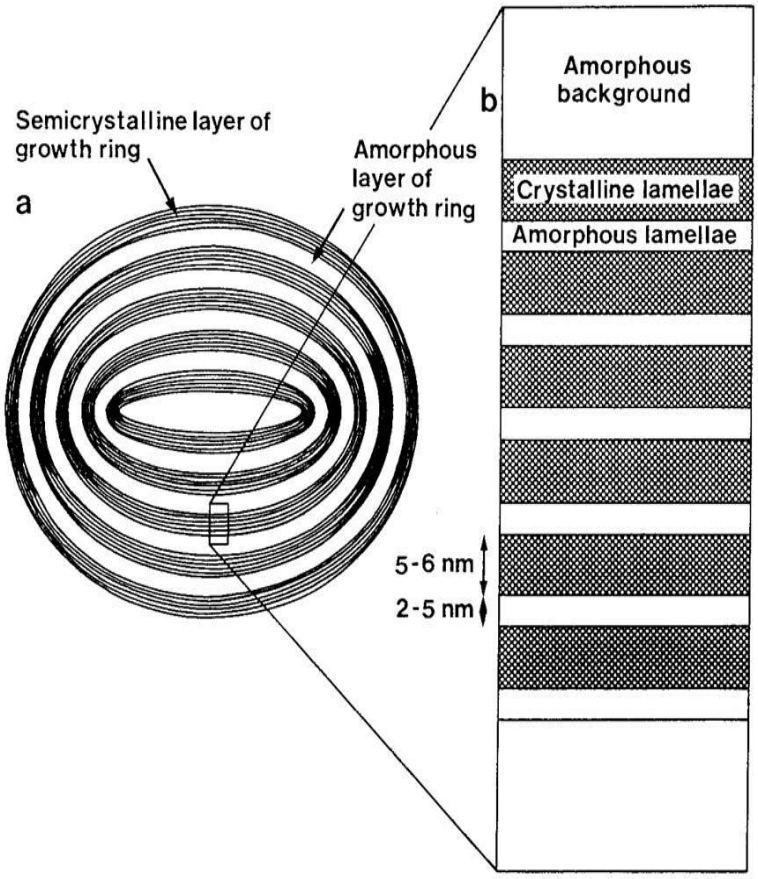


Figure 2.4 Schematic structure of starch granule.
Starch granule showing semicrystalline layers and amorphous layers is shown in (a) and a magnified view of the semicrystalline region containing alternating layers of both crystalline and amorphous lamellae is shown in (b). Adapted from Jenkins et al., 1994.

For feed and malting barley, relatively low beta-glucan concentration is required due to its adverse effect on feed energy value and malt quality. For human health, high TDF is desired because of its beneficial effects on intestinal health and prevention of diabetes and cardiovascular diseases (Chibbar et al., 2010). Balancing the concentrations of starch and non-starch components in the barley kernel will increase its utilization in feed, malt, food and industrial purposes.

2.10 Protein

Barley kernel protein concentration is an important factor for malting, food and feed quality as proteins provide energy, nitrogen and catalyze many metabolic activities during seed germination. Some minerals such as calcium, iron, phosphorus and copper are attached to barley proteins and increase their availability during utilization.

The major storage proteins in barley endosperm are hordeins, which contain 35 to 50% of total grain nitrogen depending on grain protein content (Kirkman et al., 1982). Hordeins are categorized as low-molecular-weight (LMW) hordeins (16.5 to 22 kDa) which include avenin-like proteins (A-proteins) and high molecular weight (HMW) hordeins (35 to 100 kDa) which include γ, B, C and D hordeins (Gubatz and Shewry 2011). A-hordeins are composed mainly of CM-proteins and are further grouped into CMa-1, CMb-1, CMc-1 and CMd-1 on the basis of solubility and electrophoretic mobility (Paz-Ares et al., 1982). CM-protein genes are associated with chromosomes 1H, 3H and 4H in barley (Salcedo et al., 1984). The γ hordeins are very minor components, which show mobilities similar to B hordeins on SDS-PAGE. The γ-hordeins are encoded by a locus *Hor-5* (Shewry and Parmar 1987). B- and C- hordein genes are controlled by *Hor-1* and *Hor-2* loci on the short arm of chromosome 5H, whereas D-hordeins genes are on the long arm of the same chromosome (Shewry et al., 1980). Hordeins are relatively high in glutamine, proline and asparagine, but their lysine content is low (Rastogi and Oaks, 1986). Among the essential amino acids, lysine and threonine are generally low in cereal grain including barley (Table 2.5). High lysine barley lines obtained by chemical or radiation mutagenesis produce grain with elevated lysine content (5.5%) (Ullrich, 2002).

The amount of protein accumulated in barley kernels ranges from 8-25% depending on genotype and growth conditions (Aniskov et al., 2008). In cultivated barley, the mean grain protein concentration (GPC) is 1.1 % higher in hulless varieties as compared to hulled varieties (Table 2.5) (Ullrich, 2002). For malting barley, the optimal protein concentration lies in the 9 to 12% range. Grain with lower protein concentration will produce suboptimal enzyme levels during malting followed by poor yeast growth during brewing. When the protein concentration exceeds 12%, the yield of soluble substance decreases and malt quality is lowered (Swanston and Molina-Cano 2001).

Table 2.5. Amino acid composition of hulled and hulless barley grain

	Hulled		Hulless	
Amino Acid	Mean	Range	Mean	Range
Alanine	0.44	0.11-0.56	0.47	0.16-0.67
Arginine	0.71	0.64-0.81	0.71	0.68-0.85
Aspartic acid	0.71	0.66-0.78	0.75	0.61-0.88
Asparagine	0.50	0.48-0.56	0.55	0.51-0.62
Glutamic acid	0.88	0.71-0.95	0.91	0.55-1.04
Glycine	0.42	0.32-0.53	0.44	0.37-0.53
Glutamine	0.43	0.35-0.48	0.48	0.45-0.51
Isoleucine	0.54	0.37 to 0.61	0.53	0.45-0.67
Leucine	1.11	0.84-1.18	1.16	0.93-1.21
Lysine	0.38	0.31-0.42	0.41	0.38-0.51
Methionine	0.39	0.31-0.43	0.40	0.33-0.49
Phenylalanine	0.83	0.79-0.89	0.86	0.71-0.92
Proline	1.32	1.04-1.58	1.43	1.11-1.52
Serine	0.54	0.44-0.68	0.57	0.40-0.63
Threonine	0.56	0.51-0.61	0.55	0.52-0.69
Tryptophan	0.22	0.19-0.28	0.23	0.17-0.31
Tyrosine	0.54	0.49-0.58	0.55	0.51-0.62
Valine	0.75	0.71-0.81	0.76	0.69-0.88
Total protein*	15.7	8.2-18.5	16.8	11.7-25.1

Adapted fron USDA nutrient data source (http://explore.data.gov/Health-and-Nutrition/USDA)

A long steeping time, erratic germination and haze in beer are other negative factors associated with high protein content in malting grain (Swanston and Molina-Cano 2001). In contrast to malting barley, high-protein barley (> 15%) is desired for food applications. As compared to normal barley (12% protein), the high-protein grain generally has an increased level of amylose (~27%) and β-glucan (~6%) at the expense of starch (Thomason et al., 2009).

2.11 Lipids

The lipid concentration ranges from 2.1 to 3.7% in barley grain. Hulless kernels have 1% higher lipid content than hulled kernels (Table 2.2) (Åman et al., 1985) and most of the lipids are concentrated in embryos (18% lipid concentration). Two lipid classes are recognized in grain: starch and non-starch lipids, where the former is located inside starch granules but the latter is not. Most starch lipids are phosphorylated and their concentration varies positively with amylose concentration (Morrison et al., 1993). Starch lipids have a negative effect on rate of starch hydrolysis and other grain physical properties (Qian et al., 2009). Lipid concentration has been used as a target for barley improvement and a few waxy and high amylose barley genotypes have been identified with 25% and 30% increase in lipid concentrations (Xue et al., 1997).

2.12 Minerals

The mineral (ash) content of barley kernels varies from 2.0 to 3.0%, depending on genotype. Within the seed, ash is primarily located in the embryo, aleurone and pericarp tissues (Marconi et al., 2000). As hull is rich in minerals (60 to 70%), the ash content in hulled barley is higher than in hulless barley. Despite the low mineral content, hulless barley is preferred over hulled barley for feed of monogastric animals. Minerals which affect the nutritional value for the kernel are divided into macro- and micro-elements based on concentration in foods (Table 2.6). The macro elements include calcium, phosphorus, potassium, magnesium and sodium. The rest are chloride, sulphur and silicon. Copper, iron, manganese, zinc, selenium and cobalt are the nutritionally important micro-elements in the barley kernel. Among the macro elements phosphorus and potassium are the most abundant and in terms of nutritional qualities and availability. Phosphorus in barley kernel appears as phytic acid. Monogastric animals lack the enzyme phytase for phytic acid utilization. Higher concentration of phytic acid chelates other monovalent minerals such as calcium, copper and zinc making them unavailable. Relatively

lower phytic acids are desirable for poultry feed since higher concentration results in sticky droppings.

Table 2.6. Mineral composition in hulled and hulless barley

	Hulled		Hulless	
Element	Mean	Range	Mean	Range
Macronutrient (g/100g)				
Calcium	0.05	0.03 - 0.06	0.03	0.02 – 0.04
Phosphorus	0.35	0.26 - 0.44	0.22	0.16 – 0.28
Potassium	0.47	0.36 - 0.58	0.28	0.19 – 0.41
Magnesium	0.14	0.10 - 0.18	0.08	0.05 – 0.12
Sodium	0.05	0.01 - 0.08	0.02	0.01 – 0.04
Chloride	0.14	0.11 - 0.18	0.09	0.06 – 0.12
Sulfur	0.2	0.16 - 0.24	0.12	0.08 – 0.15
Silicon	0.33	0.15 - 0.42	0.22	0.18 – 0.29
Micronutrient (mg/kg)				
Copper	6.25	2.0 - 9.0	4.2	1.5 – 3.8
Iron	45.7	36.0 - 85.0	25.0	18.1 – 29.3
Manganese	27.2	17.0 - 20.0	13.2	9.73 – 15.2
Zinc	34.4	19.0 - 35.0	21.3	17.6 – 26.4
Selenium	0.4	0.2 - 0.5	0.32	0.29 – 0.38
Cobalt	0.07	0.05 - 0.1	0.02	0.01 – 0.04

Source: Adapted with modifications from Li et al., (2000)

2.13 Determination of starch properties

2.13.1 Isolation of starch from grain

Starch physical characteristics such as peak paste time, final viscosities and swelling power can be affected by the method chosen for starch isolation (Zhong et al., 2009). The main problems are related to co-purified material such as soluble gums, proteins and lipids. A common procedure for large scale starch isolation from cereal grain is the alkali method which involves steeping ground samples in 0.03-0.05 M NaOH for 12 h followed by starch precipitation and washing. This method gives relatively good starch yield (73-85%) with minimal residual protein (0.07-0.42%) and starch damage (0.07-2.6%). However, the use of alkali causes effluent problems and affects starch pasting properties (Puchongkavarin et al., 2005). In modified versions of the alkali method, proteins and fiber are removed before starch isolation by addition of detergents and/or protease digestion under alkaline conditions (pH 10) (Lumdubwong and Seib 2000; Puchongkavarin et al., 2005). Although these modifications improve starch yields to about 95% and maintain low protein contamination (0.52%), the incubation time is long and the problems with alkali effluents remain.

For small scale laboratory research, sufficient wheat or barley starch for analysis can be isolated from samples containing one to ten embryo-less seeds (Demeke et al., (1999; Zhao and Sharp 1996). The method involves steeping seeds in water overnight at 4^{o}C followed by grinding the softened seed material into slurry. Centrifugation of slurry through 80% (w/w) cesium chloride solution is done to separate starch granules from less dense seed material. A series of washes of starch pellet with 55 mM Tris-HCl buffer (pH 6.8), water and acetone are done to remove contaminating material. Starch yield is 65-70% with minimal protein contamination.

2.13.2 Total starch determination

The iodine spectrophotometry method for quantitative starch determination is often used for rapid determination of starch concentration in plant tissues (Jarvis and Walker 2006). The original and subsequent modified methods are rapid and have high level of accuracy and are applicable both for germplasm screening and laboratory investigations. However, presence of fatty acids in starch samples can interfere with iodine absorption to starch resulting in underestimated concentrations (Karve and Kale 1990).

Another method for starch quantification is based on enzymatic hydrolysis of starch using amylo-glucosidase followed by determination of released glucose by a glucose oxidase-peroxidase-chromogen system (Dekker and Richards 2006). A drawback of this method is that starch gelatinized in water at 130 ^{o}C before enzymatic hydrolysis will lead to underestimated values of starch concentration. However, if the starch is solublized in alkali and subsequently neutralized with acetic acid a more accurate value of starch concentration is obtained as the alkali solution inhibits one or more of the enzymes involved in subsequent glucose analysis. The precision of the method is increased by the use of purified β-glucanase- and cellulase-free amyloglucosidase (McCleary et al., 1997).

2.13.3 Amylose determination

The amount of amylose in starch is an important factor for starch characterization as amylose to amylopectin ratio affects starch physicochemical properties such as gelation temperature and rate, solubility and retrogradation characteristics. The first methods developed for amylose determination were based on the ability of amylose to form helical inclusion complexes with iodine. The blue amylose/iodine complexes have a maximum absorption at 620 nm, whereas complexes between amylopectin and iodine are reddish with maximum absorption at 540 nm. Potentiometric (Banks and Greenwood, 1975), amperometric (Larson et al., 1953) or spectrophotometric (Morrison and Laignelet 1983) techniques are used to quantify the amount of amylose / iodine complexes. However, lipid complexation with iodine and amylose retrogradation in solution can lead to underestimated values of amylose concentration (Sargeant, 1982). Another problem are formation of blue complexes between long chains on amylopectin and iodine (Sargeant 1982), resulting in an overestimation of amylose concentration.

The large difference in the number of non-reducing end-groups between amylose and amylopectin can be utilized for amylose determination (Gibson et al., 1997). In this method, lectin concanavalin A (Con A) is used to form precipitates with non-reducing end-groups. The Con A / amylopectin complexes are removed by centrifugation and amylose concentration is determined by measuring the carbohydrates remaining in the supernatant. The method does not require standard curves and any mathematical calculations, however regular optimization of pH, temperature and ionic strength for full precipitation of amylopectin molecules is required to avoid amylose overestimation (Matheson and Welsh 1988; Demeke at al., 1999).

Amylose content in starches either in debranched or in native form can be determined by the size exclusion chromatography (SEC) (Sargeant, 1982). In this protocol, amylose and amylopectin are separated based on differences in their hydrodynamic radius and excellent resolution of amylose and amylopectin can be obtained with 2.6 (i.d) x 79 cm long Sepharose CL-2B columns (Wang et al., 1993). However, the method is time consuming and only a small number of samples can be analysed per day. Recent methods for amylose determination are based on digestion of starch with debranching enzymes followed by fractionation of released glucans by high-performance size-exclusion chromatography (HP-SEC). To prevent amylose retrogradation during glucan separation, the debranched starch is dissolved in DMSO. The eluate is monitored by a refractive index detector and the relative concentrations of amylose and amylopectin are calculated by integrating peak area generated from chromatographs (Demeke et al., 1999). The two most commonly used debranching enzymes in the procedure are pullulanase and isoamylase. Isoamylase is preferred due to its ability to debranch amylopectin completely to yield linear chains (Atwell et al., 1980).

2.13.4 Amylopectin chain length determination

Amylopectin chain length determination is important because of its relation to starch crystalline structure and starch functionality. The analysis also provides information about the various steps in the starch biosynthetic pathway as activity and specificity of many starch biosynthetic enzymes can alter amylopectin fine structure (Wang et al., 2003). Methods previously used for estimation of chain length distribution of amylopectin included HP-SEC with a differential refractometer and high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD). Amylopectin chain-length distribution obtained from these methods are weight-based, however calibration of columns will allow indirect molar-based determinations (Hanashiro et al., 2002). The chain-length distribution obtained from HP-SEC is bimodal where the first fraction (F_1) is made up of long B chains, whereas F_2 fraction contains short B and A chains (Hizukuri 1985). The correlation between the weight-average chain length and the ratio of the two weight fraction (F_2 / F_1) are positive. The observation implies that average chain lengths are dependent on amount of the two fractions. The separation method suffers from the limitation of separating individual chains and chain lengths greater than dp 26.

The high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC–PAD) is used to analyse homoglucans, α-(1→4)-glucans up to dp 60 (Shi and Seib 1995). Like HPSEC, weight based measurements can be done by HPAEC–PAD. Matrix-assisted laser desorption / ionization mass spectrometry (MALDI-TOF-MS) originally developed for mass determination of large molecules such as proteins can also be applied to carbohydrates (Wang et al., 1999). The method requires little sample purification and provides quick measurements with more detailed information about molecular weight of individual chains as compared to HPAEC-PAD (Broberg et al., 2000).

Another method for determination of glucans generated through debranching of amylopectin involves attachment of a fluorescent tag to the molecules followed by fractionation (O'Shea and Morell 1996). The original method used a DNA sequencer attached to a fluorescence detector for sample analysis. In recent methods, the reductive amination of reducing end with charged fluorophore 8-amino-1,3,6-pyrenesulfonic acid (APTS) is followed by direct on-line measurement of chain-length distribution of molecules on molar-basis is possible by pre-column labelling. Method is successful with longer oligosaccharides (dp > 80) which cannot be resolved with either HPSEC or HPAEC-PAD. Further improvement of this method to increase the sensitivity and higher resolution (dp ≥ 100) is the use of fluorophore-assisted carbohydrate electrophoresis (FACE) (Morell et al., 1998).

2.13.5 Starch analysis by atomic force microscopy (AFM)

Atomic force microscopy (AFM) is a non-destructive method for imaging biological materials such as nucleotides, starch granule morphology and amylopectin fine structure (Kirby 1996; Liu et al., 2001; Limanskii 2007). It uses a scanning probe for three-dimensional imaging of samples either in dry or liquid environments with nm and Å lateral and vertical resolutions respectively (Marti et al., 1987). Samples usually imaged under dry conditions have thin layer of water covering the surfaces. Imaging mode can be contact and dynamic or alternating contact (AC) mode. Contact mode is not suitable for biological samples due to poor resolution arising from physical interaction of sharp cantilever tip and sample. However, Marszalek et al., (2001) measured the force required in induced conformational changes in starch biopolymers using AFM with a cantilever sharp tip. For biological samples the dynamic or alternating contact (AC) modes are the most useful tools. The AC mode can be fully contact or intermittent contact where

magnetic force applied causes cantilever to oscillate vertically producing a gentle force between the tip and sample. Starch sample preparation and deposition methods affect imaging and results. From diverse botanical origin many polysaccharide molecules including starch have been imaged by AFM (Kirby et al., 1996) using drop deposition or the aerosol spray method (Liu et al., 2001). To prevent starch aggregation before imaging samples are kept at gelatinization temperature prior to deposition. Mica has a hydrophilic surface and a smooth area hence it is the choice material for AFM sample deposition and imaging. Comparison of different deposition methods proved aerosol spray as the best method for fine images (Kirby 1996). Measurements obtained from AFM images include contour length and height (nm), linear mass density (M_n), average molecular weight (M_w), polydispersity index (M_w / $M_{n)}$, and degree of polymerization (dp). These parameters which relate with starch physical properties affecting starch hydrolysis are different for amylose and amylopectin molecules.

2.13.6 Resistant starch (RS)

Englyst et al., (1982) coined the term 'resistant starch' (RS) to include starch fractions that remained undigested with amylase/pullulanase unless the sample is first solubilised with 2 M potassium hydroxide or DMSO. Based on this assertion, food starch is classified as either glycemic or resistant (Englyst and Cummings 1985). Glycemic starch is hydrolysed in the digestive tract and is classified as rapidly digestible starch (RDS) or slowly digestible starch (SDS). While RDS releases glucose within 20-30 min, SDS releases glucose slowly with digestion completed within 20-110 min. RS is categorised into four groups based on different reasons for enzyme resistance (Table 2.7). The first group of resistant starch (RS1) is physically protected whole or partly milled grains, seeds and legumes. In this category resistance starch is reduced by milling and/or chewing. RS2 is obtained from raw potatoes, green bananas, some legumes and high amylose starches in ungelatinized starch granules. Reduction of RS content in RS2 is by food processing. Retrograded starch is termed as RS3, where cooked and cooled food products from potatoes, bread or corn flakes is exposed to repeated moisture and heat treatment. The final category of resistant starch is chemically modified starch with cross-linking reagents such as ethers and esters. The most common type of resistant starch is RS3 because starchy foods are cooked in excess water and allowed to cool before serving (Ratnayake and Jackson 2008). The amount of resistant starch in RS3 correlates with amylose concentration (Cai and Shi 2010).

Table 2.7. Classification of resistant starches

Type	Description	Food sources	Resistance reduced by:
RS1	Physically protected	Whole or partly milled grains and seeds, legumes and pasta	Milling, chewing
RS2	Ungelatinized granules with B-type crystallinity that are slowly hydrolyzed by alpha amylase	Raw potatoes, green bananas, some legumes, high amylose starches	Food processing and cooking
RS3	Retrograded starch	Cooked and cooled potatoes, bread, corn flakes, food products with prolonged and/or repeated moist heat treatment	Processing conditions
RS4	Chemically modified starches due to cross bonding with chemical reagents, ethers, esters etc.	Some fiber-drinks, foods in which modified starches have been used (eg certain breads and cakes)	Less susceptible to digestibility *in vitro*

Reproduced with modifications from Nugent, 2005.

Starch and other carbohydrates that escape digestion in the small intestine are fermented in colon to produce short-chain fatty acids (SCFA) such as acetic acid, butyric acid and propionic acid (Topping and Clifton 2001). SCFA are the preferred substrates by cells lining colon (colonocytes). Butyric acid is reported to improve colon health by serving as energy source for colon epithelium cells and also suppresses proliferation of cancerous cells (Hamer et al., 2010). Clinical research has suggested that consumption of food high in RS3 decreases the factors associated with metabolic syndrome (obesity, diabetes, hyperlidpidemia, and hypertension) and

may prevent or treat other chronic diseases (Topping and Clifton 2001). Cereal crop (barley) improvement programs which produce high amylose starch genotypes will end up with RS3 foods for lowering of glycemic index, prevention and control of diabetics and hypocholesterolemia (Goňi et al., 1997)

2.13.7 Measurement of RS

Methods for determination of RS in foods and food products have increased over the years due to growing awareness of physiological and nutritional benefits of RS on human health. The physiological significance of RS was fully realized by early 1990s by which time several methods were developed during the European research program (EURESTA). According to the definition of resistant starch as the sum of starch and products of starch degradation that are not absorbed in small intestine of healthy individuals, analytical methods should measure all starch and α-dextrins present. Data from any analytical method must also be validated by comparison of *in vitro* generated data with *in vivo* data obtained from healthy subjects (Champ et al., 2003). Determination of RS based on physiological definition has certain drawbacks: (1) because in any starch based foods, RS content is reported as a function of starch structural organization and physiological and functional state of environment during digestion (Goňi, et al, 1996). Two main *in vivo* methods used for RS determination are (a) Intestinal intubation and collection of residual starch at the end of the ileum in healthy subjects (b) The ileostomy model using subjects with a large-bowel resection but an intact and healthy small bowel. These two human-based methods give large variations in RS as subjects react differently to food and food products. Variations observed in previously mentioned methods have necessitated the use of *in vitro* method that mimics conditions in subjects.

Several *in vitro* methods have been used to quantify RS in foods and food products (Englyst and Hudson 1996; McCleary and Monaghan 2002; Muir et al., 1995). The main principles behind these methods were: (1) enzymatic solubilisation of starch within 120 min of incubation with α- amylase and amyloglucosidase at 37 ^{o}C; (2) extraction of solubilised products with 80% (v/v) ethanol and discarded; (3) solubilisation of pellet (RS) with 2 M KOH and amyloglucosidase; and (4) measurement of the released glucose using glucose oxidase. McCleary and Monaghan (2002) reviewed parameters affecting RS determination, such as α-amylase concentration, pepsin pre-treatment, pH of incubation mixture, amyloglucosidase

inclusion, shaking and stirring of the reaction mixture during incubation and RS recovery from pellet. Results from the study led to pepsin pre-digestion omission, incubation together of both pancreatic α-amylase and amyloglucosidase at pH 6·0 and alcohol precipitation. Pellet is dissolved in 2 M KOH followed by hydrolysis of RS by amyloglucosidase and glucose measured by using the GODPOD reagent (glucose oxidase-peroxidase reagent; Megazyme International Ireland Ltd, Wicklow, Ireland). The current procedure is accepted as the standard RS determination method by AOAC-2002.02 (Champ et al., 2003).

2.13.8 Factors affecting starch hydrolysis and RS

The susceptibility of starch to hydrolysis by α-amylase is dependent on the botanical origin of starch, starch physical characteristics (e.g. granule type), physical characteristics of food, presence of other nutrients and anti-nutrients, amylose / amylopectin ratio, retrogradation of amylose and α-amylase (Srichuwong et al., 2005). Starch digestibility is related to crystalline polymorphic forms (Srichuwong et al., 2005). Jane et al., (1997) reported that starch with A-type X-ray diffraction (cereal starches) is more susceptible to amylolysis than those ith a 'Bw' type pattern (tuber starches). A-type starch crystallites with short double helices and inferior crystallites are readily digested by α-amylase (Jane et al., 1997). The fraction of crystalline structures (Planchot et al., 1997), amylose concentration (Stevnebø et al., 2006), starch granule sizes and shapes, association between molecules and number of pores on starch granule surfaces (Jane et al., 1997) affect rate of starch hydrolysis.

Starch granule structure (shape and size) is one of the most important starch properties which affect rate of enzyme hydrolysis. It has been demonstrated through electron microscopy that α-amylase attack starts on starch granule surface and continues hydrolyzing from within a granule after penetration (Li et al., 2004). The rate of starch hydrolysis correlates with starch granule size. Large and small starch granules have negative and positive correlations respectively with rate of enzymatic starch hydrolysis Stevnebø et al., 2006). Small starch granules (size $\leq$ 10 µm) have larger surface area for enzyme action compared to large starch granules (size $\geq$ 15 µm). Also the higher amylose and lipid concentrations associated with large starch granules compared to small granules (Gao et al., 2009) explains the relative ease of hydrolysis in the latter.

Amylopectin chain length (A and B chains) responsible for starch crystalline structure affects rate of enzymatic hydrolysis. Waxy starch amylopectin has shorter chain lengths (dp 23-29) compared to high amylose starch (dp 30-40) (Jane et al., 1997). Studies by (Fuwa et al., 1997) demonstrated that source of hydrolytic enzyme affects rate of starch hydrolysis rate. The pancreatic α-amylase is more efficient in starch hydrolysis compared to amyloglucosidase, isoamylase and bacterial, fungal and barley malt α-amylases (Fuwa et al., 1997).

2.13.9 Relationship between RS and starch composition and structure

Resistant starch (RS) in foods and food products is made up of retrograded starch, physically inaccessible starch, starch-nutrient complexes, chemically modified starch and starch that is indigestible due to enzymatic inhibition (Englyst and Macfarlane, 1986). The RS amounts in foods can be altered by food processing or plant breeding (eg. high- or low-amylose variants of cereals and grains). Starch gelatinization results in loss of molecular order of starch granules and makes starch easily digestible. Retrograded starch forms during cooling by partial re-association of crystalline structure resulting in formation of RS3 (Eerlingen et al., 1994). The X-ray diffraction pattern of retrograded starch shows B-type crystallinity (Jane et al., 1997) which is more resistant to enzyme hydrolysis. The rate of retrogradation and amount of RS3 is correlated with amylose, temperature and heating cycles (Szczodrak and Pomeranz 1991). High amylose maize and wheat starches retrograded faster compared to normal and waxy maize (Russel et al., 1989). RS3 in retrograded starch is made up of mainly retrograded amylose. Compared to retrograded normal wheat starch, high amylose starch was 9.8 times higher in RS amount (Jia et al., 2007). Amylopectin degree of polymerization (dp) affects starch structure and is dependent on genotype and environment (Holtekjølen et al., 2008).

Waxy starches contain relatively greater proportion of outer short branches (dp 14-18). In contrast to waxy starch, the dp of amylopectin in high amylose starch is composed mainly of intermediate and long chains (dp < 18) (Jia et al., 2007). The limited dimensions of the chains reduce stability of crystallites to enzyme hydrolysis in amylopectin compared to amylose (Russel et al., 1989). The result was the observed slow crystallization after gelatinization of amylopectin process which takes days or weeks compared to amylose.

Starch granules sizes affect RS level as potato (Franco and Ciacco, 1992) and high amylose maize and wheat starches (Jia et al., 2007) are known to be very resistant *in vitro* and

incompletely absorbed *in vivo* compared to most cereal starches. The sizes and shapes of starch granules in these starches were large A-type and irregular respectively. Protein (Escarpa et al., 1997), enzyme inhibitors in seeds such as polyphenols, phytic acid and lectins (Thompson and Yoon 1984), ions (Escarpa et al., 1997) and lipids (Czuchajowska et al., 1991) affect RS in foods and food products. While protein and iron concentration was found to negatively correlate with RS, enzyme inhibitors and lipids correlated positively with RS. Amylose-lipid association prevented re-association behaviour of amylose upon retrogradation of starch and increases RS (Czuchajowska et al., 1991). The conclusion based on reports from previous studies is that RS in starch is a factor of both extrinsic and intrinsic conditions. Amylose concentration, amylopectin degree of polymerization and granule morphology correlated with RS (Jia et al., 2007).

2.14 Starch Biosynthesis: Mutations and phenotypes

2.14.1 Starch biosynthetic enzymes in plants

Green plant chloroplasts have the unique ability to harvest light energy to fix reduced carbon dioxide and water into a simple carbohydrate backbone $(CH_2O)_n$ through the process of photosynthesis. The photosynthetic reactions result in a number of triose and hexose phosphates including glucose-1-phosphate. ADP-glucose pyrophosphorylase (AGPase) converts glucose-1-phosphate and ATP to ADP-glucose which initiates starch biosynthesis in leaves. Chloroplasts synthesize transitory starch during the day, and degrade accumulated starch at night (dark period) to form maltose, glucose, hexose phosphates and triose phosphates. Some of these products are converted into the major transport sugar, sucrose, which is transported to storage tissues such as seeds, tubers or roots, where it is through a series of biosynthetic reactions converted into storage starch (Fig 2.5). Studies of plant mutants have revealed a core set of enzymes involved in starch biosynthesis are encoded by conserved genes in the plant kingdom (Table 2.8). As outlined in Fig. 2.5, the key enzyme activities include ADP-glucose pyrophosphorylase, (AGPase; EC 2.7.7.23), starch synthase (SS; (EC 2.4.1.21), starch branching enzyme (SBE; α-(1→4)-glucan 6-glucosyl-transferase, EC 2.4.1.18), starch debranching enzyme (DBE; EC 3.2.1.41 and EC 3.2.1.68), starch phosphorylase (Pho1) and disproportionationg enzyme (D) (see reviews by Zeeman et al., 2001; Ball and Morell 2003, and references therein). A minimum set of starch biosynthetic

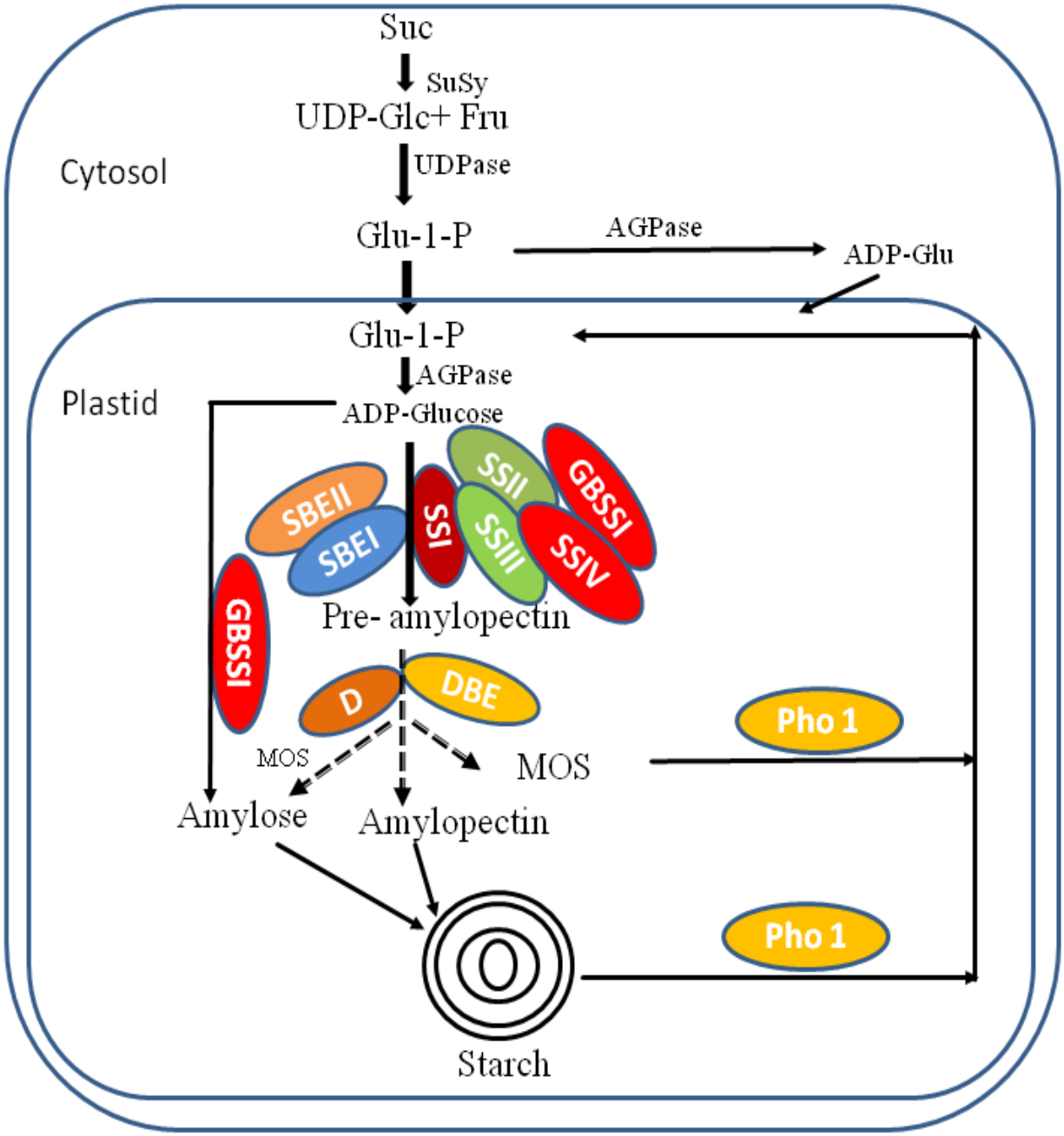


Figure 2.5 A model for starch biosynthesis in plants.
The model is based on reviews by Ball and Myers 2005; Tetlow et al., 2004. The abbreviations used for starch biosynthetic enzymes are: Sucrose (Suc), Sucrose Synthase (SuSy), UDP-glucose (UDP-Glc), Fructose (Fru), UDP-glucose pyrophosphorylase (UDPase), ADP-glucose pyrophosphorylase (AGPase), Glucose-1-phosphate (Glu -1-P), MOS (malto-oligosaccharide).

Table 2.8. Mutations affecting storage starch composition and structure in crop species

Crop	Mutations	Affected enzyme	Starch phenotype	Reference
Barley	Risǿ 6	Cytosolic AGPase	Low starch	Johnson et al., 2003
Barley	*Lys5*	Plastidal AGPase	Low starch	Patron et el., 2004
Maize	s*hrunken-2*	Lacks L-AGPase	Low starch, high sucrose	Michaels & Andrew, 1986
Maize	*brittle-2*	Lacks S-AGPase	Low starch, high sucrose	Michaels & Andrew, 1986
Pea	*Rb*	Low AGPase activity	Low starch, high sucrose	Smith et al. , 1989
Barley	*low amylose*	Reduced GBSSI activity	Low starch or no amylose	Patron et al., 2002
Wheat	*waxy*	"	"	Chibbar & Chakraborty 2005
Maize	*Waxy*	"	"	Tsai, 1974
Rice	*Glutinous*	"	"	Sano, 1984
Pea	*low amylose*	"	"	Denyer et al., 1995
Barley	*sex6*	Lacks SSII activity	Increased amylose/ low starch	Morell et al., 2003
Wheat	*null Sgp-A1, B1, D1*	"	"	Yamomori et al., 2000
Maize	*sugary-2*	"	"	Zhang et al., 2004
Pea	*rugosus-5*	"	"	
Barley		Lacks SBEI/II activity	Increased amylose/ amylopectin ratio	Regina et al., 2010
Rice	*amylose extender*	"	"	Nishi et al., 2001
Wheat		"	"	Regina et al., 2009
Maize	*amylose extender*	"	"	Yun et al., 1993
Pea	*Rugosus*			
Barley	*Riso-17/Notch-2*	Lacks ISA	Reduced starch/ high phytoglycogen	Burton et al., 2002
Maize	*sugary-1*	"	"	James et al., 1995
Rice	*sugary-1*	"	"	Rahman et al., 2005

Adapted from Jeon et al., (2010) and Nakamura (2002) with modifications.

enzymes include two AGPase, five SS, three SBE and two DBE. Thirteen of the various isoforms of starch biosynthetic enzymes are homologous in all plants characterized to date (Morell and Myers, 2005). Also photosynthetic organisms with very small genomes such as green algae produce a full set of starch biosynthetic enzymes implying each isoform has a role that cannot be adequately compensated by another form (Morell and Myers, 2005). However, the isoforms show functional differences within plant species, organs, sub-cellular localization, substrate specificity and interaction with other enzymes.

Starch biosynthetic enzymes are more or less starch granule-bound or entirely soluble in the stroma of plasmids (Tetlow et al., 2004a). Two types of granule-bound enzymes are recognized; (1) those with loose association to granule and easily removable by protease digestion and / or detergent buffer containing a reducing agent (Denyer et al., 1993: Rahman et al., 1995) and (2) tightly granule-bound proteins that are only extracted upon gelatinization of starch in SDS buffer containing a reducing agent. Starch granule bound proteins from starch granules can be distinguished as 150 polypeptides on a two-dimensional SDS-PAGE gel (Borén et al, 2004). Key starch biosynthetic enzymes such as GBSSI, SSI, SSII and SBEIIb are integral proteins, of which GBSSI and SBEIIb appear to undergo proteolysis within the granule. Hordein B and D, serpin Z4 and pyruvate orthophosphate dikinase are other proteins found attached to the granule surface.

2.14.2 ADP-glucose pyrophophorylase (AGPase)

The first committed step in the production of transitory starch in chloroplast and storage starch in amyloplast is the production of glucose donor ADP-glucose (ADP-Glc). The reaction is rate-limiting and catalyzed by AGPase (Okita, 1992) as follows:

α-D-glucose-1-phosphate + ATP $\rightarrow$ ADP-glucose + PPi.

AGPase in higher plants is a heterotetrameric enzyme with two large subunits (AGPase-L) and two small catalytic subunits (AGPase-S) (Villand et al., 1992). The small subunits are relatively conserved between species, whereas large subunits are more divergent (Georgelis et al., 2007). Two distinct small subunits of AGPase destined to plastids and cytosol, respectively, are produced in wheat and barley due to alternative splicing of the same transcript (Burton et al., 2002b; Thorbjørnsen et al., 1996). AGPase is an allosterically regulated enzyme (Ghosh and Preiss 1966) and the level of control depends on the species and tissue (Ball and Preiss, 1994).

The photosynthetic product 3-phosphoglyceric acid (3-PGA) activates the enzyme and inorganic phosphate (PPi) causes enzyme inhibition (Doan et al., 1999) and this control is stronger in leaves as compared to endosperm (Sikka et al., 2001).

Mutations in either of the AGPase subunits result in altered starch phenotype. The *Shrunken-2* and *Brittle-2* mutants of maize (Hannah and Nelson 1976) have lesions in the large and the small subunits of the cytosolic AGPase, respectively. The kernels of s*hrunken-2* mutant are sweet due to high sucrose levels, deeply dented, and contain opaque floury endosperm with 25% reduced starch content (Hutchisonc, 1921). The fragile seeds of *brittle-2* mutant germinate poorly and are dark and shrunken due to 25-34% lower starch content (Preiss et al., 1990). An AGPase mutant in barley, *Risø 16*, has a deletion in the small cytosolic AGPase subunit resulting in 44% lower starch production and 28% seed weight loss (Johnson et al., 2003). The possibility to elevate starch content in seeds by increasing the AGPase activity has been demonstrated by expressing an unregulated form of ADP-glucose pyrophosphorylase from *E. coli* in potato (Stark et al., 1992), maize (Hannah, 2005; Wang et al., 2007), rice (Sakulsingharoja et al., 2004) and wheat (Smidansky et al., 2002; 2003). In tuber crops, the starch increase results in large tubers (Lloyd et al., 1999), whereas seed number is increased in cereals, possibly due to enhancement of sink strength and reduced seed abortion (Hannah and James 2008). These observations suggest that increased starch production in crops can be achieved with the use of cultivars with unregulated ADP-glucose pyrophosphorylase.

2.14.3 Starch synthases (SS)

ADP-glucose (ADP-Glc), produced by AGPase, is utilized in plastids as a glucose donor for starch synthesis by SS. The enzymes catalyze transfer of the glycosyl moiety of ADP-Glc to a non-reducing end of a preexisting α-(1→4) glucan chain (Tsai, 1974). The reaction is as follows:

$$(1, 4 - \alpha\text{-D-glucosyl})_n + \text{ADP-glucose} \rightarrow (1, 4 - \alpha\text{-D-glucosyl})_{n+1} + \text{ADP}$$

The SSs belong to GT5 of the glycosyltransferases super family carrying a catalytic core composed of conserved starch catalytic and glycosyltransferase1 domains respectively (Carbohydrtae-Active enzymes database (CAZY). Glycosyltransferase1 domains are found among proteins that transfer ADP, UDP, CMP or GDP-linked to glycogen, lipopolysaccharides and fructose-6-phosphate substrates (Yep et al., 2004). The structure of SS is characterized by

alternating α-helixes (H1-H8) and β-sheets (E1-E7) at both the N- and C-terminal domains. The two domains are separated by a linker that makes the protein flexible (MacGregor 2002). Amino acid residues that reside on the helices and beta sheets are highly conserved and motifs such as K-X-G-G-L and are involved in catalytic and substrate binding activities (Furukawa et al., 1993; Nichols et al., 2000).

Plants have five classes of SS and each class is often represented by several isoforms. The GBSS forms (GBSSI and GBSSII) are entirely starch granule-bound (Denyer et al., 2001), SSIII and IV are found mostly found in stroma (Fujita et al., 2006), whereas SSI and II are distributed between stroma and the starch granule (Denyer et al. 1995; Dai, 2010). All the SSs are characterised by an amino-terminal transit peptide (cTP) followed by a N- terminal domain both of which are variable and specific for each class (James and Myers, 2008) and a catalytic domain at the C-terminal end (Senoura et al., 2007). Similarities and differences exist among SSs in domains involved in substrate binding and catalytic activity which influence the structure of glucan polymer produced. Only ADP-Glc is used by SSI, SSI and SSIII as substrate, whereas both ADP-Glc and UDP-Glc are utilized by GBSSI (Leterrier et al., 2008; Shapter et al., 2009). The SS genes of cereals differ in the number of exons and introns and their lengths. A comparison of *Gbss1, SsI* and *Ss2a* from barley and *Ss3* and *Ss4* from the closely related species wheat is shown in Fig. 2.6. The *Gbss1* gene of barley contains 12 exons, of which the first exon is untranslated. The barley *Ss1* gene has 15 exons, but only eight exons are carried by *Ss2a*. As compared to wheat *Ss2a*, the second intron of barley *Ss2a* is split by a small intron resulting in its exons described as 2a and 2b. *Ss3* and *Ss4* of wheat contain 16 exons (Fig 2.6). Barley SSI (Gubler et al., 2000) and SSII (Morell et al., 2003) are encoded by genes on chromosome 7HS whereas SSIII and SSIV which is closely related to each other are encoded on the long arm of chromosome 1 (Leterrier et al., 2008).

2.14.3.1 Role of SS in amylopectin biosynthesis

The complex amylopectin biosynthesis involves activities of SSs, SBEs, and DBEs (Fig. 2.5), and the relative contribution of each enzyme depends on plant species and tissue (Smith, 2001). The soluble SSI is responsible for elongation of linear chains up to a length of 6-15 glucose units (James et al., 2003), but the enzyme has been shown to be dispensable for starch biosynthesis in rice (Fujita et al., 2006). Two genes encode SSII in monocots, *SS2a* and *SS2b*,

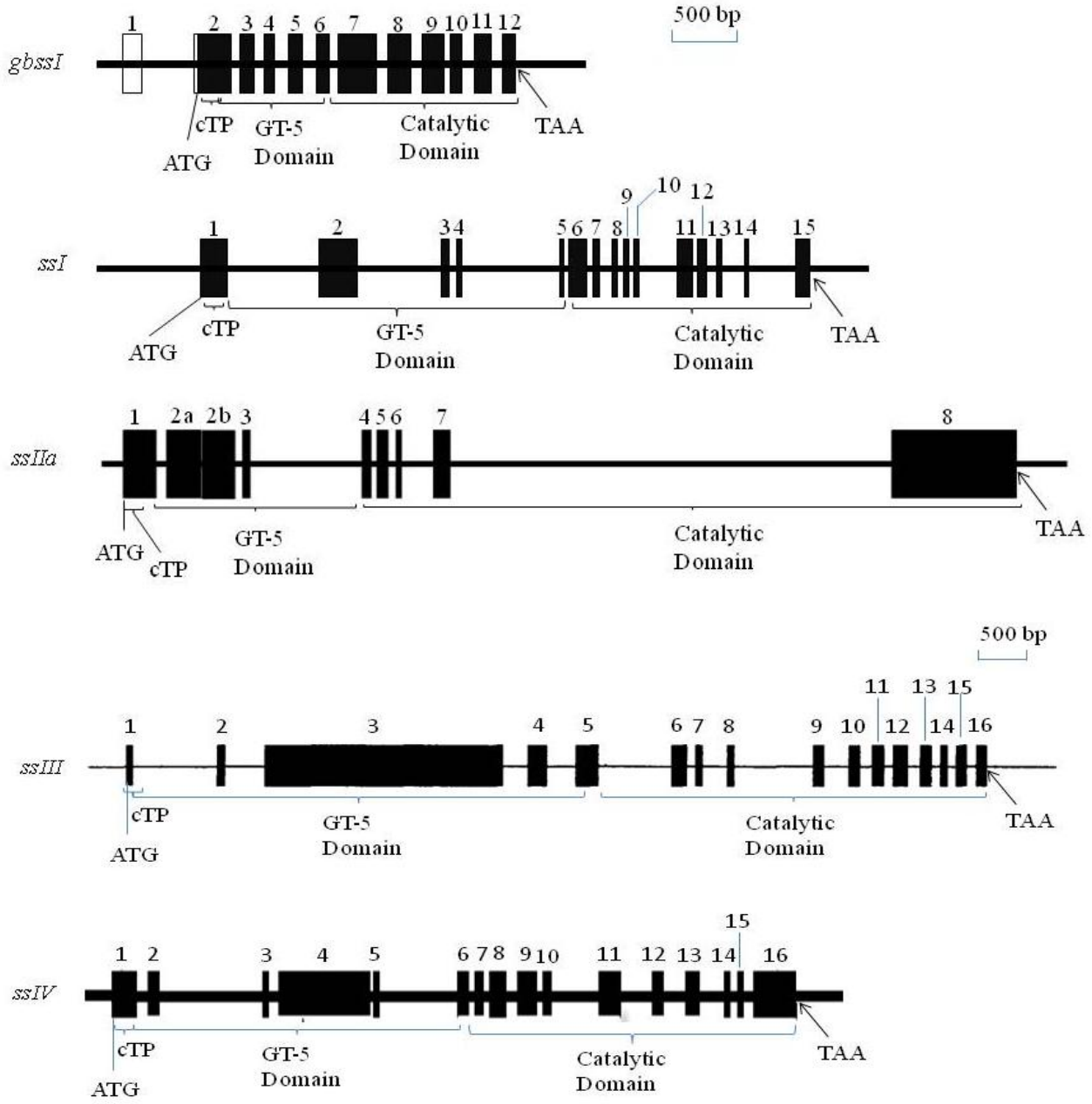


Figure 2.6 Schematic structure of SS genes in cereals.
Exons are represented by vertical blocks and introns by horizontal line. Location of translation initiation (ATG) and stop (TAA) codons are shown. Exons encoding transit peptides (cTP), glycosyltransferase-5 domain (GT-5) and catalytic (glycosyltransferase I; GT-1) domain are indicated. (Figure from Rohde et al., 1988; Leterrier et al., 2008).

and they have possibly evolved from a gene duplication event (Gao and Chibbar, 2000). Each SSII isoform plays a specific role in elongation of amylopectin branches. Mutations in *Ss2* genes of pea (*rug5*; Craig et al., 1998), maize (*sugary-2*; Zhang et al., 2004), wheat (Konik-Rose et al., 2009; Yamamori et al., 2000) and barley (*sex6*; Morell et al., 2003) result in reduced starch concentration and crystallinity, altered starch granule morphology, lower starch gelatinization temperature and a higher concentration of amylose in grain starch. The *Ss2* mutation affects amylopectin fine structure, where fewer intermediate chains are produced, but the relative number of short (dp < 10) and long chains (dp > 25) are increased. Antisense inhibition of *Ss2*expression in potato increases the relative amount of amylopectin chains with dp 8-12, lowers the gelatinization temperature and viscosity of starch, but does not affect starch granule morphology (Edwards et al., 1999).

Mutations eliminating SSIII activity in maize are known as *dull* (Gao et al., 1998) and are accompanied by a reduction in SBEIIa activity (Gao et al., 1998). Other pleiotropic effects caused by *Ss3* mutations include inactivation of *waxy* and *amylose extender (ae)* genes or *sugary1* encoding isoamylase (Garwood et al., 1976). These effects impact amylopectin synthesis, resulting in modified chain-length distribution and decreased starch synthesis. Analysis of amylopectin produced by *Arabidopsis thaliana* suggests that SSIII catalyzes synthesis of amylopectin chains that extend between clusters (Szydlowski et al., 2009). The starch synthase IV (SSIV) class of starch synthases appears in expressed sequence tag (EST) databases from a range of species including *Arabidopsis*, cowpea, wheat, and *Chlamydomonas*. However the identification of the corresponding protein has yet to be demonstrated. Yamamori et al., (2000) suggested that SSIV does not have a role in starch biosynthesis, whereas Roldan et al., (2007) proposed a role in production of short glucan chains and starch granule initiation in *Arabidopsis thaliana.*

2.14.3.2 Synthesis of amylose by GBSSI

Two forms of GBSS are produced in cereals: the 60 kDa GBSSI encoded from waxy locus on chromosome 7HS (Rohde et al., 1988) and the ~59 kDa GBSSII encoded from group 2 chromosomes (Nakamura et al., 1998; Vrinten and Nakamura 2000). Although GBSSI and II share same the enzyme nomenclature (EC 2.4.1.242), they are only 69% identical at the amino acid level (Shapter et al., 2009). GBSSI is produced in endosperm tissue during seed

development, in photosynthetic tissues and in pollen of cereals. GBSSII is accumulated in leaf, culm and pericarp tissues, it is not found in endosperm and thus does not significantly contribute to final starch concentration in mature grain (Vrinten and Nakamura, 2000). It has long been known from plant mutants that GBSSI deficiency results in low amylose or amylose-free starch (Tsai, 1974). In wheat and other polyploid species, the number of functional GBSSI alleles show a good correlation with the amount of amylose produced (Mangalika et al., 2003). Some waxy genotypes produce a GBSSI variant with very low affinity for ADP-glucose as revealed by *in vitro* enzyme assays (Delrue et al., 1992). Besides elongation of amylose chains, GBSSI extend preexisting amylopectin chains (Denyer et al., 1999a, 1999b) and participates in the buildup of B-type crystals that interconnect amylopectin clusters in Chlamydomonas starch (Fulton et al., 2002; Wattebled et al., 2002). It is not clear whether GBSSI converts existing amorphous amylopectin into a crystalline structure or catalyzes direct synthesis of a semi-crystalline product.

2.14.4 Starch branching enzyme

Branching of amylose and amylopectin molecules is catalyzed by SBE, which belong to the α-amylase family (Jespersen et al., 1993). The members of the α-amylase family share a common structural motif composed of eight β-strands surrounded by eight α-helices running parallel to each other to form an $(\beta/\alpha)_8$-barrel domain, which has catalytic and substrate-binding properties (MacGregor, 1993). SBE cleaves α-(1→4) bonds and re-attaches released chains onto existing amylose or amylopectin chains by forming an α-(1→6) branch point. The created new branch provides a new non-reducing end for chain elongation by SS. There are two classes of SBE in plants, designated SBEI and SBEII based on biochemical and physicochemical properties (Rahman et al., 2001). Isoforms of SBE differ in terms of spatial and temporal expression (Fisher et al., 1993; Sun et al., 1998; Radchuk et al., 2009).

2.14.4.1 Starch branching enzyme I (SBEI)

Studies of amylopectin produced by SBEI mutants suggest SBEI is responsible for transfer of longer glucan chains that link multiple clusters of amylopectin with middle sized chains located in the amorphous lamellae (Satoh et al., 2003b). Branches introduced by SBEI are primarily located on less branched polyglucans with dp ≥ 16. In contrast to studies in rice, Blauth at al., (2002) observed no effect on amylopectin chain length profile in a *Sbe1* mutant in maize.

No mutants for *Sbe1* are known for barley. Starch produced by a *Sbe1* mutant in rice shows higher resistance to lower onset of both urea and thermo-gelatinization (Satoh et al. 2003b). The observation was consistent with the effect of a higher proportion of long chains of amylopectin on gelatinization. In most plants, one isoform of SBEI is present; however, wheat, barley, rye and triticale also produce a larger SBEI isoform, SBEIc (152 kDa) (Båga et al., 2000). This isoform is preferentially associated with large A-type starch granules in cereals showing bimodal starch granule size distribution (Peng et al., 2000).

2.14.4.2 Starch branching enzyme II (SBEII)

SBEII in cereals is represented by two distinct isoforms: SBEIIa and SBEIIb encoded by different genes (Fig. 2.7) which have specific roles during amylopectin synthesis (Gao et al., 1996; Nakamura 2002; Sun et al., 1998). *Sbe2a* is ubiquitously expressed in all cereal plant tissues (Ohdan et al., 2005), whereas *Sbe2b* is primarily expressed during endosperm development (Nakamura (2002). Rice and maize *Sbe2a* mutants show no effect on amylopectin chain length profiles in transient starch but a slight reduction in chain lengths $6 < dp > 8$) in short chains of endosperm starch is noted (Satoh et al. 2003a). SBEIIa is believed to support the function of SBEI in leaves where *Sbe2b* is not expressed (Satoh et al. 2003a).

Lesions in *Sbe2b* (amylose extender gene, *ae*) in maize results in increased amylose concentration (Yun and Matheson 1993) and amylopectin with more long and less short chains (Kubo et al., 2008). Downstream regulation of either *Sbe2a* or *Sbe2b* alone in wheat (Regina et al., 2006) does not affect starch synthesis (amylose concentration), however a combined suppression of both enzymes results in increased amylose (> 70%) in starch. Similarly in potato, a knockout of *Sbe1* alone had no effect on amylose concentration however down-regulation of both *Sbe1* and *Sbe2* together resulted in increased amylose concentration (Schwall et al., 2000). For barley, there are no known natural *SbeII* mutations, which could be used for starch modification and diversification of starch uses.

2.14.5 Starch debranching enzymes (DBE) and amylopectin biosynthesis

Isoamylase (glycogen 6-glucanohydrolase; ISA) and pullulanase (pullulan 6-glucanohydrolase; β-limit dextrinase; PUL) are two DBE implicated in starch biosynthesis and determination of the final amylopectin structure (Ball et al., 1996; Nakamura, 1996). The two

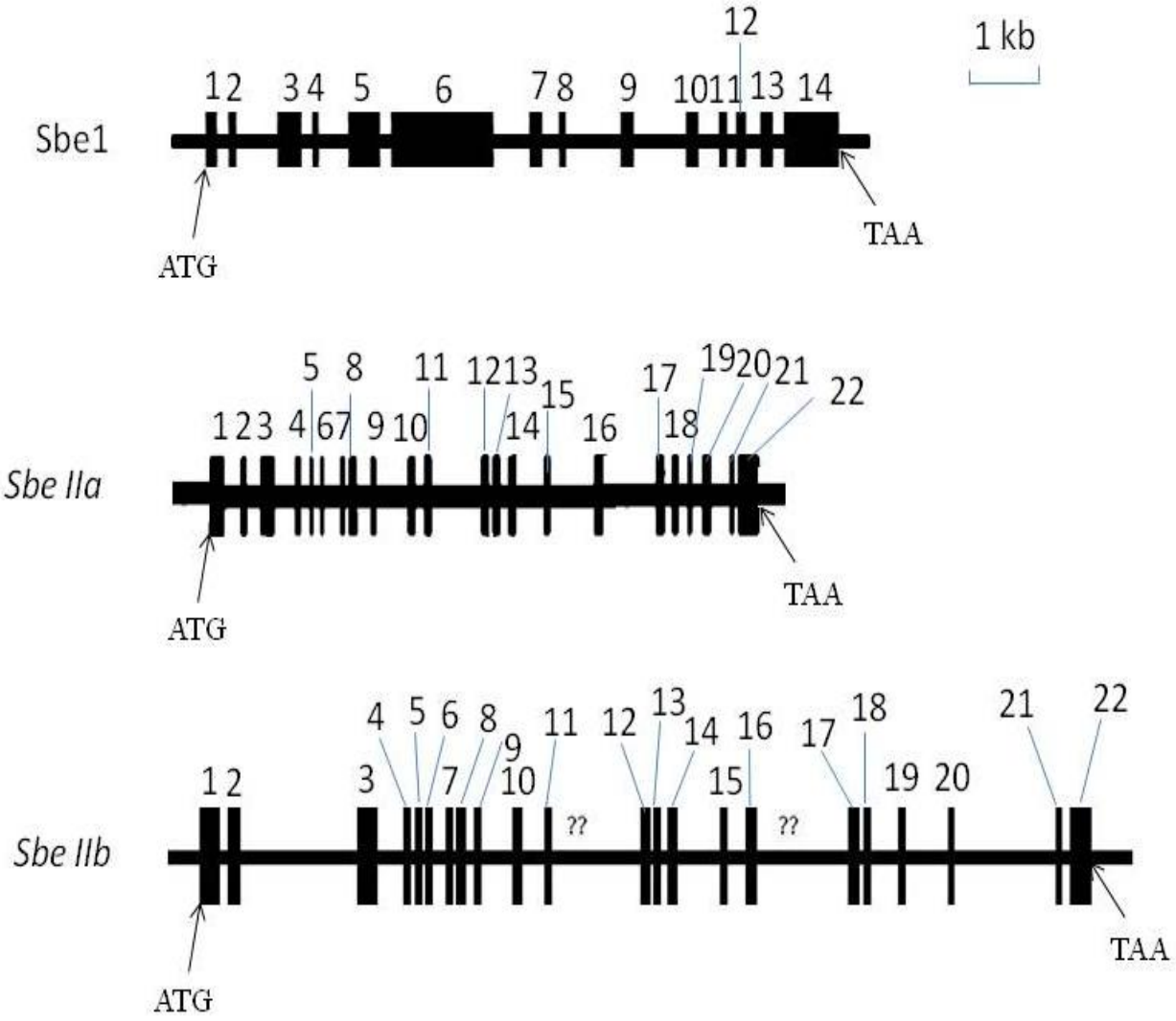


Figure 2.7 Schematic illustration of SBE genes.
Vertical blocks represents coding regions and horizontal bars are non-translated regions. Location of translational start (ATG) and stop (TAA) are indicated. (Sun et al., 1998, 2003)

enzymes hydrolyze α-(1→6) branches but differ with respect to their substrate specificity. PUL effectively debranches α-(1→6)-linkages in pullulan, a polymer mainly made up of maltotriose groups linked by α-(1→6)-linkages. ISA debranches glycogen and amylopectin, but not pullulan (Nakamura et al., 1996). There are three ISA genes and one PUL gene identified in plants (Burton et al., 2002; Dinges et al., 2003).

During amylopectin biosynthesis DBE trims improper branches of amylopectin introduced by SBE, which allows the resultant amylopectin to crystallize within the starch matrix (Ball et al., 1996; Nakamura, 2002). In the absence of DBE activity, sugars and a highly branched glucan polymer, phytoglycogen, accumulate instead of starch granules (Mouille et al., 1996; Zeeman et al., 1998). Depending on the DBE mutation, mild sugary-amylopectin to severe phytoglycogen is produced in maize *sugary 1* (Pan and Nelson 1984; James et al., 1995; Dinges et al., 2001), barley *Risø 17* and *Notch-2* mutants (Burton et al., 2002), and rice *sugary 1* (Fujita at al., 2003; Nakamura et al., 1996). The ISA gene mutation carried by *sugary-1* mutants causes pleiotropic effects on several starch biosynthetic enzymes (Dinges et al., 2001), where the net effect is an increase in the number of A-chains on amylopectin at the expense of long (DP > 37) chains (James et al., 1995; Nakamura et al. 1996). Besides amylopectin biosynthesis, the barley ISA has been implicated in starch granule initiation (Burton et al., 2002). The barley ISA gene is induced by a sugar responsive WRKY transcription factor, SUSIBA2, produced only in the endosperm (Sun et al., 2003). *Pul* activities are high during seed germination when the enzyme has an important function for starch hydrolysis (Burton et al., 1999; Dinges et al., 2003).

A lower level of *Pul* expression is seen during seed development (Sissons et al., 1993; Burton et al. 1999), but the role of PUL for starch biosynthesis is not well understood. *Pul* mutants in rice produce starch with have shorter of DP $\leq$ 12 chains of amylopectin, whilst double mutants of *Pul/Sul1* recorded even higher short chains DP $\leq$ 7 (Fujita et al., 2009). Multiple effects on starch biosynthesis, such as lower amylose/amylopectin ratio, fewer small granules and lower starch yield, are seen when limit dextinase activity is increased in barley (Stahl et al., 2004).

2.14.6 Starch phosphorylase

Plants contain two types of starch phosphorylase: *Pho1* and *Pho2*, which catalyze the transfer of glucosyl units from Glc-1-P to the non-reducing end of α-(1→4)-linked glucan chains. *Pho1* is localised in plastids whereas *Pho 2* is cytosolic. *Pho1* is believed to act on starch granule surface, where it modifies starch structure phosphorolytically providing Glc-1-P substrate which is recycled back into the starch biosynthesis pathway (Tetlow et al., 2004). Tickle et al., (2009) observed an increase in *Pho1* activity alongside BEI, BEII, and the SS proteins during grain development in wheat and suggested a role for it in starch biosynthesis.

Pho1 mutants of rice accumulate smaller starch granules with shrunken endosperm and shorter chains of amylopectin of dp ≤ 11. The synthesis of linear glucans (dp 4-6) from maltooligosaccharides by *Pho1* in the presence of Glc-1-P is believed to act as primers in glucan initiation (Satoh et al., 2008).

2.15. Formation of protein complex between starch biosynthetic enzymes

Starch biosynthetic enzymes act in a highly coordinated manner to produce amylopectin which is architecturally conserved in different plant starches. It has been observed that mutations at one locus in starch biosynthetic pathway have pleiotropic effects on other starch biosynthetic enzymes. For example, in maize *ae* mutant lacks SBEIIb, but SBEI activity is absent or reduced and properties of an isoamylase type DBE are altered (Colleoni et al., 2003). Similarly in maize, mutations which affect pullulanase (*zpu-204*) or isoamylase (*su1-st*) type DBE cause a reduction in SBEIIa activity, although SBEIIa protein amount is not reduced (James et al., 1995; Dinges et al., 2001; 2003). In endosperm of *ae* rice (lacking SBEIIb), SSI activity is also significantly reduced (Nishi et al., 2001). In rice and barley *Ss2* mutants, loss of SSII activity abolishes the binding of SSI, SBEIIa and SBEIIb within the granule matrix, although no loss of affinity of these enzymes to amylopectin or starch is observed (Morell et al., 2003; Umemoto and Aoki 2005) which suggests the formation of enzyme complexes during starch biosynthesis.

Using isolated wheat amyloplasts, it has been shown that some of the key starch biosynthetic enzymes form protein complexes that are dependent upon their phosphorylation status (Tetlow et al., 2004b). Similar heteromeric complexes made up of different combinations of starch biosynthetic enzymes have also been identified in maize (Hennen-Bierwagen et al., 2008). The concept of starch biosynthetic enzymes acting in complexes, which are dependent upon the phosphorylation status, adds another level of control in starch biosynthetic process. Recently, a proteomics study showed that phosphorylation of GBSSI, SBEIIb and Pho1 is needed for their incorporation into starch granules (Grimaud et al., 2008).

2.16 A model for amylopectin biosynthesis in cereal endosperm

The synthesis of amylose is solely the responsibility of GBSSI, whereas amylopectin synthesis occurs by a concerted action of several starch biosynthetic isozymes in cereal endosperm. Many models have been proposed to explain amylopectin biosynthesis which include the cluster model (French, 1972), the glucan trimming model (Ball et al., 1996) and the

water soluble polysaccharide-clearing model (Zeeman et al., 1998). The more acceptable model: the glucan trimming model, consist of glucan initiation, starch amplification, starch branching and debranching (Fig 2.8). However, the mechanisms involved in glucan initiation process remain to be resolved. Pho1 is believed to extend the initial priming sites of free chains of malto-oligosaccharides in the presence of Glc-1-P. This is supported by significant expression of *Pho1* at initial developmental stage alongside other starch biosynthetic genes (Ohdan et al., 2005). This preposition is still open to debate since the high ratio (> 50) of the plastidal concentration of Pi to Glc-1-P against the equilibrium constant of 2.4 does not favour Pho1 activity.

In the glucan-trimming amylopectin biosynthesis model, starch synthases elongate the primed glucan in amylopecting molecule up to a critical stage where branching enzyme introduces α-(1→6) branches (Fig 2.8). Branching only occurs when the glucan attains a critical size to interact with branching enzymes catalytic sites. The optimal molecular size and catalytic sites required for the introduction of branch points by starch branching enzymes is as shown in Figure 2.8 C. Branching results in phytoglycogen-like structures due to improper introduction of branch points which are trimmed immediately by debranching enzymes. The debranched molecules become perfectly spaced branches in amylopectin allowing for repeated processes.

The soluble polysaccharide-clearing model involves *Pho1* in starch biosynthesis. *Pho1* is speculated to degrade malto-oligosaccharides (MOS) generated by debrabching activity of ISA during amylopectin synthesis or through phosphorolytic degradation of starch at the surface of the granule (Fig 2.7). The resulting Glc-1-P thereby is re-introduced into the starch synthesis pathway. Problems with this model include the almost complete loss of starch in *Pho1* rice mutants (Satoh et al., 2008) and the high Pi to Glc-1-P ratio in plastids (> 50) above the equilibrium constant (2.4).

2.17 Need for barley kernel starch improvement

Most of the world production of barley is utilized as feed (65%) or production of malt (33%) and only 2% is consumed as human food (Baik and Ullrich, 2008). Due to barley's high nutritional value, there are good opportunities to increase production of barley for human consumption. Although, the specific parameters for food barley genotypes have not been outlined, the preferred grain should be rich in minerals and complex carbohydrates (starch and

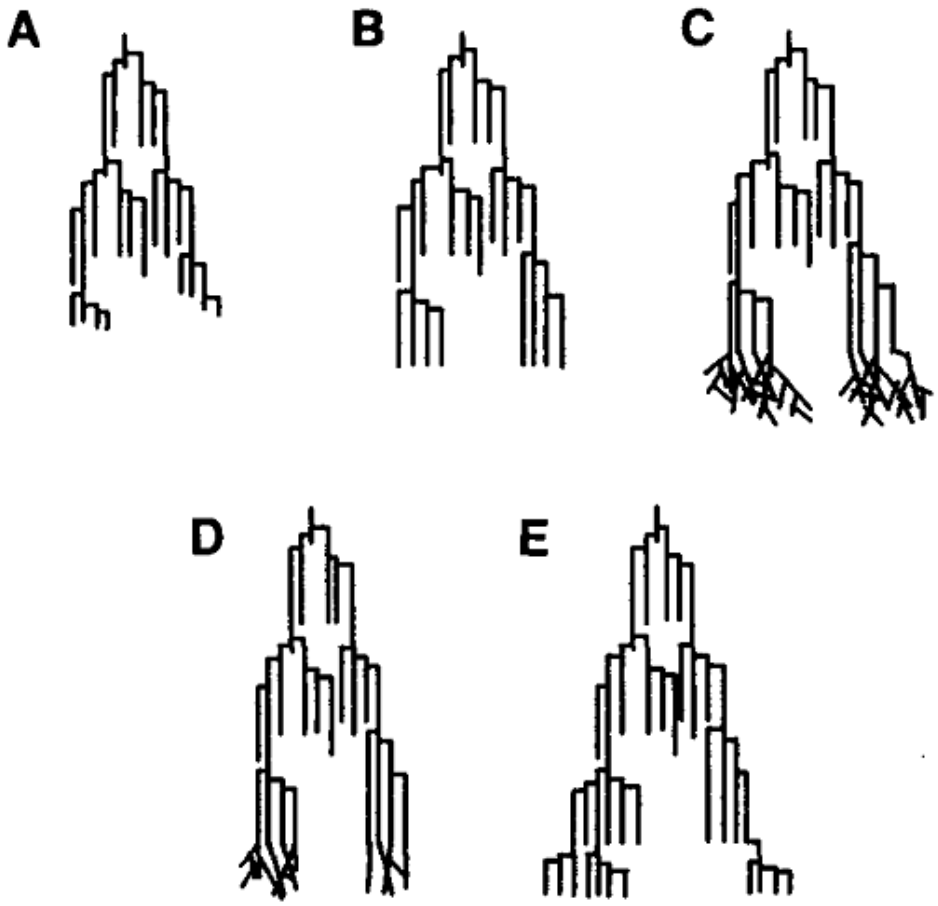


Figure 2.8 Glucan-trimming model of amylopectin biosynthesis.
A trimmed amylopectin molecule ready to be further elongated is shown in (A). Various SSs elongate the amylopecting molecule up to a critical stage where the minimum chain length for SBE action is attained (B). SBE isoforms introduce (α-1→6) branch points to produce phytoglycogen-like molecule in (C). The action of DBEs trims the branches to generate perfectly spaced chains in amylopectin (D) that will allow for further synthesis. (Adapted from Mouille et al., 1996).

non-starch), contain a well-balanced protein and amino acid composition and have low fat content. Increased grain protein (> 15%) in barley kernel is also desired; however most barley genotypes grown today have a low to moderate protein concentration because high protein concentration has an adverse effect on malting and beer quality (Swanston and Molina-Cano 2001).

Of special interest for food uses is a high dietary fiber (β-glucan) content, which has a preventive effect on heart diseases and type-2 diabetes by lowering blood cholesterol and blood

glucose levels and aiding weight loss in humans (Izydorczyk and Dexter, 2008). High amylose content is another desirable grain character as long glucan chains generate increased amounts of resistant starch during digestion. Resistant starches stimulate production of short chain fatty acids which is reported to be beneficial for colon health and prevention of colon cancer (Jenkins et al., 1998). Both waxy and increased amylose barley genotypes are relatively high in both β-glucan and dietary fiber compared to normal genotypes, but grain yield can be low in these genotypes (Izydorczyk et al., 2000). The connection between β-glucan biosynthesis and starch accumulation in grain is not known, but is believed to involve common factor(s) regulating carbon partitioning.

High amylose starch has a current use in food thickening, deep frying (less oil absorption) and some industrial applications, whereas waxy starch is preferred for enhanced stability and prolonged shelf-life of frozen foods, paper-making and adhesives (Zheng and Sosulski, 1998). As waxy starch phenotype is a result of GBSSI inactivity, increased amylose concentrations are due to other mutations in starch biosynthetic genes. Amylose : amylopectin ratios in starch can be manipulated by altering GBSSI and SBEII (*waxy / amylsose extender*) activity in wheat (Lafiandra et al., 2010; Sestili et al., 2010; Regina et al., 2006), maize (Jiang et al., 2010) and rice (Wei et al., 2010). In barley, very high amylose concentrations were obtained by RNAi mediated inhibition of *Sbe2a* and *Sbe2b* genes (Regina et al., 2010). Inactive SSIIa (*sex6* mutation) in barley and SSII isoforms in wheat also lead to increased amylose phenotypes (Morell et al., 2003; Yamamori et al. 2000). Thus, there are many examples how amylose / amylopectin ratios in starch can be altered in plants, but these alterations / mutations often show negative effects on plant performance and grain yield and do not immediately allow commercial production. An increased understanding of how starch biosynthesis in leaves and production of storage starch in sink tissues is interconnected with plant development and seed production is needed. To further our basic knowledge on how these grain quality traits interact, nine barley genotypes with different levels of β-glucan, starch composition and digestion profiles were analyzed in this study. The study led to the identification of candidate genes for some of the mutant phenotypes analyzed.

CHAPTER 3

BARLEY GRAIN CONSTITUENTS, STARCH COMPOSITION AND STRUCTURE AFFECT STARCH *IN VITRO* ENZYMATIC HYDROLYSIS

3.1 Abstract

The relationship between starch physical properties and enzymatic hydrolysis was determined using ten different hulless barley genotypes with variable carbohydrate composition. The ten barley genotypes included one normal starch (CDC McGwire), three increased amylose starch (SH99250, SH99073 and SB94893), and six waxy starch (CDC Alamo, CDC Fibar, CDC Candle, Waxy Betzes, CDC Rattan and SB94912). Total starch concentration positively influenced thousand grain weight (TGW) ($r = 0.70$, $p < 0.05$). Increase in grain protein concentration was not only related to total starch concentration ($r = -0.80$, $p < 0.01$) but also affected enzymatic hydrolysis of pure starch ($r = -0.67$, $p < 0.01$). However an increase in amylopectin unit chain length between DP 12-18 (F - II) was detrimental to starch concentration ($r = 0.46$, $p < 0.01$). Amylose concentration influenced granule size distribution with increased amylose genotypes showing highly reduced volume percentage of very small C-granules (< 5 µm diameter) and significantly increased ($r = 0.83$, $p < 0.01$) medium sized B granules (5-15 µm diameter). Amylose affected smaller (F-I) and larger (F-III) amylopectin chains in opposite ways. Increased amylose concentration positively influenced F-III (DP 19-36) fraction of longer DP amylopectin chains (DP 19-36) which was associated with resistant starch (RS) in meal and pure starch samples.

The rate of starch hydrolysis was high in pure starch samples as compared to meal samples. Enzymatic hydrolysis rate both in meal and pure starch samples followed the order waxy > normal > increased amylose. Rapidly digestible starch (RDS) increased with a decrease in amylose concentration. Atomic force microscopy (AFM) analysis revealed higher polydispersity index of amylose in CDC McGwire and increased amylose genotypes which could contribute to their reduced enzymatic hydrolysis, compared to waxy starch genotypes. Increased β-glucan and dietary fiber concentration also reduced the enzymatic hydrolysis of meal samples. An average linkage cluster analysis dendrogram revealed that variation in amylose concentration significantly ($p < 0.01$) influenced resistant starch concentration in meal and pure starch

samples. RS is also associated with B-type granules (5-15 µm) and amylopectin FIII (19-36 DP) fraction. In conclusion, the results suggest that barley genotype SH99250 with less decrease in grain weight in comparison to that of other increased amylose genotypes (SH99073, SH94893) could be a promising genotype to develop cultivars with increased amylose grain starch without compromising grain weight and yield.

3.2 Introduction

Barley (*Hordeum vulgare* L.) cultivation and utilization ranks fourth after maize, rice and wheat (FAO, 2008). However, barley is the least utilized cereal for human food consumption. It is poor man's food in underdeveloped countries. In contrast to this, in western countries it is fast becoming a part of natural healthy diet (Newman and Newman, 2004). Barley is an excellent source of complex carbohydrates and β-glucans, two important constituents of dietary fibre. This has led to the wide acceptance of barley as human food with significant human health benefits (Blakeney and Flinn, 2005; Oscarsson et al., 1996; Rosin et al., 2002; USA-FDA, 2006). A growing number of health conscious consumers have increased demand for foods such as barley. Starchy food after ingestion is assimilated in the upper gastrointestinal tract. A variable portion of starch, not assimilated in the upper gastrointestinal tract, classified resistant starch (RS) reaches the large intestine where its fermented products, primarily short chain fatty acids (SCFA), help in maintaining healthy viscera (Wei et al., 2010). In addition, SCFA also lower lumen pH creating a less conducive environment for cancer and other diseases (Topping and Clifton, 2001).

Digestion of starchy foods is a complex process, affected by the rate of starch digestion and absorption, including source of food material, its components, physical nature, presence of enzyme inhibitors, antinutrients and processing methods (Goñi et al., 1997; O'Dea et al., 1980). Rate of carbohydrate absorption in fiber rich food is low due to high viscosity created in the upper digestive tract (Englyst and Kingman, 1990; O'Dea et al., 1991). It is believed that enzymes responsible for carbohydrate hydrolysis are excluded by the fiber components in food thereby preventing / slowing the rate of hydrolysis (O'Dea et al., 1980).

On the basis of plant source and processing methods, RS is classified into four major types. RS type 1 is trapped in plant architecture helping escape from amylolysis. Physical damage *via* chewing or milling makes them accessible. RS type 2 is derived from specific plant

sources such as green banana or starch with poor gelatinization and hence slower hydrolysis (e.g. high amylose corn starches). Increase in the amylose content of starch granules favours increase in dietary fiber and resistant starch content (Brown, 2004). RS type 3 is formed via retrogradation after cooking and RS type 4 includes chemically modified starches (Yao et al., 2009).

In addition to 60-80% of carbohydrates in barley grain, it also contains 9-13% nitrogenous compounds, 1-2% fat and 10-15% water (Chibbar et al., 2004). The predominant carbohydrate in barley grain is starch and it ranges from 62 to 77% of the grain dry weight (Bhatty and Rossnagel, 1998). Starch physicochemical properties and end use are significantly affected by the amylose to amylopectin ratio and other storage compounds (Izdorczyk, 2000; Hang et al., 2007). On the basis of amylose concentration, barley starch can be classified into normal (~25- 27% amylose), waxy (non-detectable to < 5% amylose), and increased amylose (> 35% amylose) (Bhatty and Rossnagel, 1992; 1998; Zheng et al., 1998; Izdorczyk, 2000). Increased amylose starch food is not completely digested in the small intestine of monogastric animals and is, therefore, classified as resistant starch (Topping and Clifton, 2001; Higgins, 2004). Waxy starch (95 to 100% amylopectin) finds its use in the food industry for improvement of properties including uniformity, stability, texture and better freeze-thaw ability of the food products (Chibbar and Chakraborty, 2005).

Starch hydrolysis by α-amylases is influenced by various physical and structural features including granule size; phosphorus content; complexes between amylose and lipid (Crowe et al., 2000); distribution and perfection of crystalline region in both amorphous and crystalline lamellae (Zhang et al., 2008); starch crystallinity and packing (Jane et al., 1997); porosity; structural inhomogeneity and degree of integrity (Copeland et al., 2009); connectivity between hilum and surface channels (Kim and Huber, 2008); interaction of amylopectin chains during hydrolysis and extent of helix formation in amylose and amylopectin (Haralampu, 2000; Miao et al., 2009). Inhibition of α-amylases by maltose and maltotriose may also be relevant (Colonna, 1988). During cooking, starch is gelatinized and amylose molecules are leached out of the swollen starch granules as coiled polymers which on cooling associate as double helices and form hexagonal networks (Haralampu, 2000; Jane and Robyt, 1984). In waxy starches instead of this network, aggregate formation occurs, which is more susceptible to hydrolysis by amylases

(Miao et al., 2009). In brief, the factors that hide starch from amylases contribute to resistant starch content.

Amylopectin chain length distribution (CLD) and packing have been reported to play an important role in starch digestibility. In increased amylose rice, RDS is reported to be strongly correlated with short chains whereas RS is associated with long and intermediate chains fraction (Benmoussa et al., 2007). On the contrary, amylopectins of increased amylose barley varieties did not show significantly larger proportions of long chains than that of normal and waxy barley starch (Song and Jane, 2000). In Cassava longer chains of amylopectin form complexes with amylose and contribute to starch high gelatinization group (Charles et al., 2005). Srichuwong and coworkers (Srichuwong et al., 2005) analysed starches from different botanical sources and reported no significant correlation between functional properties of starch with average granule size, shape or apparent amylose content but strong correlation with amylopectin CLD. In cereals both amylose and amylopectin contribute towards SDS formation (Zhang et al., 2006).

Endogenous and externally added lipid and proteins also affect starch digestibility and resistant starch formation (Miao et al., 2009). Complexes with long chain fatty acids are more resistant than complexes with shorter chains as amylose becomes less available to form network aggregates (Putseys et al., 2010).

The differences in starch composition and structure can be utilized in food applications to reduce the risk of diabetes and/or digestive tract related diseases. In the present study, grain composition and starch properties were analyzed from ten different hulless barley genotypes with varying amylose concentration, an important feature for RS formation. The major objective of the study was to study the influence of grain constituents and starch properties on rate of *in vitro* enzymatic hydrolysis of barley pure starch and meal samples with varied amylose concentration.

3.3 Materials and Methods

3.3.1 Plant material

The study was conducted with ten hulless barley (*Hordeum vulgare* L.) genotypes, including one normal starch (CDC McGwire), six with waxy starch (CDC Candle, CDC Alamo, CDC Rattan, CDC Fibar, SB94912 and waxy Betzes) and three with increased amylose concentration (SH99250, SH99073 and SB94983). Three replicates of all the genotypes were grown in standard small plots at Kernen Crop Research Farm, University of Saskatchewan,

Saskatoon, Canada, in 2005, except CDC McGwire which was grown in 2003 and waxy Betzes grains were taken from long term storage. One thousand seeds from each genotype were weighed for thousand grain weight (TGW) determination. Ten grams (10 g) of barley seed from each replicate in duplicate were ground though a 0.5 mm sieve by UDY mill (UDY Corporation, Fort Collins, CO). All determinations were reported on dry weight basis.

3.3.2 Protein concentration

Protein concentration was determined by the combustion method with the FP-528 Protein/Nitrogen Analyzer (LECO Corporation, St Joseph, MI). Meal samples (0.25 g ± 0.01 g) in duplicates were combusted for protein analysis. Percent protein concentration of samples was obtained using the formula %P = %N x C, where C is 5.7 for wheat while 6.25 for all other crops (AACC method 46-10).

3.3.3 Crude lipid concentration

Crude lipid concentration was determined by Goldfisch lipid extraction method, using hexane as the extraction solvent (AACC method 30-25). Barley flour (2 g) was placed in Whatman #2 filter paper, folded into a lipid extraction thimble and then clamped into the lipid extractor (Goldfisch, Labconco Corporation, Kansas City, MO). Hexane (60 mL) was added to each pre-weighed beaker and connected to the extractor. The extraction was carried out for 5 h. The extracted lipid left behind in beaker after hexane recovery and drying at 100^{o}C for 30 min was cooled and weighed. Percent lipid (% lipid) was expressed as weight of lipid per gram dry weight of initial material used.

3.3.4 Beta-glucan determination

β-glucans were determined using enzymatic (AACC method 32-23; McCleary and Codd, 1991) as well as the flow injection method using Calcoflour and fluorescent detection (Aastrup and Jørgensen, 1998).

For the enzymatic analysis, barley meal (100 mg) suspended in ethanol (50% v/v, 1 mL) was mixed with sodium phosphate buffer (5 mL; 20 mM pH 6.5) and boiled for 5 min with an intermittent vortexing. After cooling it was digested with 200 units of lichenase (1000 U/mL, Megazyme, International Ireland Limited, Co. Wicklow, Ireland) for 1 h at 40 ºC. Total volume was adjusted to 30 mL with water. An aliquot (0.1 mL) taken in triplicate was mixed with 0.1

mL of sodium acetate buffer (50 mM, pH 4.0) in the first tube while 4 units of β-glucosidase (40 U/mL, Megazyme) was added to the rest, followed by incubation at 40 ^{o}C for 15 min. The reaction mixture was treated with 3 mL of glucose oxidase/peroxidise (GOPOD) reagent and re-incubated for 20 min. The glucose control is used as a single calibration point standard curve. The amount of glucose was determined by comparing it against a glucose control at 510 nm using a spectrophotometer (DU800, Beckman Coulter, Brea, CA) and values were used to estimate β-glucan concentration in samples (Li et al., 2008).

Flow injection analysis (FIA) uses calcofluor as the binding probe. A concentration range (0- 200 µg in 100 µL water) of barley meal for each sample and a series of standards [0-1.75 µg of barley β-glucan (Sigma-Aldrich St. Louis, MO) in 100 µL] were prepared. Phosphate buffer (0.1 M, pH 8.0) was used as carrier and for Calcoflour preparation. The working solution (100 µL) containing 35 mg/L Calcofluor in 0.01% Triton X-100 was rapidly added to each sample including the standards, mixed and injected at a flow volume of 2.0 mL/min using an auto-sampler. Increase in fluorescence, proportional to the β-glucan concentration was determined by comparing sample peak area or peak height with a standard curve generated from injections of β-glucan standards (Li et al., 2008).

3.3.5 Total dietary fiber (TDF)

Dietary fiber was determined by sequential enzymatic digestion of barley flour (1 g) with thermo stable α-amylase (3,000 U/mL, Megazyme International Ireland Ltd., Wicklow, Ireland) in 50 mL of 0.08 M phosphate buffer pH 6.0 at 95 ^{o}C, followed by acidification and redigestion for 30 min in 35 units of protease (350 U/mL tyrosine) at 60 ^{o}C (AACC method 32-21). The final digestion step consisted of treatment with 40 Units of amyloglucosidase (200 U/mL Megazyme) for 30 min at 60 ^{o}C. Dietary fiber was finally precipitated with 4 volumes of ethanol (95% v/v), washed with 78% (v/v) ethanol and acetone and air dried. One part was used for protein determination by the Kjeldahl method and the other for ash determination (incinerate for 5 h at 525 °C). Total dietary fiber was weight of residue less the weight of protein and ash (Prosky et al., 1988).

3.3.6 Total starch concentration

Total starch concentration was determined on the basis of AACC approved method (AACC method 76-13)**.** In brief, 100 mg (duplicate) samples of ground barley was weighed into

10 mL glass tubes and dispersed in 2 mL, 80% (v/v) ethanol. To each sample, 3 mL of α-amylase (240 U/mg, Megazyme) in 50 mM MOPS [3-(N-morpholino) propanesulfonic acid] buffer (pH 7.0) was added. Samples were vortexed and incubated in a boiling water bath for 8 min with 3 times of intermittent shaking. Samples were allowed to cool at room temperature and reincubated (50 ^{o}C, 30 min) with 330 Units of amyloglucosidase (3300 U/mL, Megazyme) in 4 mL of sodium acetate buffer (200 mM, pH 4.5). After the reaction was complete, sample volume was made up to 100 mL with distilled water and three aliquots (100 µL) were transferred into different test tubes with 3 mL of glucose determination (GOPOD) reagent. Samples in duplicates were incubated including glucose standards at 50 ^{o}C for 20 min. Total starch concentration was determined as free glucose by measuring the absorbance at 510 nm (Hucl and Chibbar, 1996). Starch concentration was calculated on a percent dry weight basis (McCleary et al., 1997).

3.3.7 Starch extraction

Coarsely ground barley meal from 4 to 6 grains was steeped in 0.02N HCl (2 mL) overnight at 4°C. After neutralization with 0.2N NaOH and centrifugation at 4000 g, the residue was crushed in 2 mL of 0.1 M Tris-HCl buffer (pH 7.0) containing 0.5% $NaHSO_3$, followed by enzyme treatments, proteinase (15 U/g barley), lichenase (2 U/g barley) and β-xylanase (8 U/g barley) (You and Izydorczyk, 2002). Samples were digested overnight and filtered though a 100 µm pore size nylon filter. Crude starch slurry was centrifuged for 10 min at 4000 g. The precipitate was re-suspended with 200 µL of water, layered over 1 mL of 80% (w/v) cesium chloride solution and centrifuged at 13,000 g for 30 min. The starch pellet was washed twice with water followed by acetone washing and overnight air drying.

3.3.8 Starch granule size distribution

Starch granule size distribution (by volume) of the starch slurries was determined using a laser diffraction particle size analyzer (Mastersizer 2000, Malvern Instruments, Malvern, England). A 40 mg/mL starch solution was used for size analysis at a pump speed of 1700 rpm.

3.3.9 Amylose concentration determination

Amylose concentration was determined as described by (Demeke et al., 1999) with a high performance size exclusion liquid chomatography (HPSEC). One milligram of gelatinized starch (1mg) was incubated for 4 h (40 ^{o}C) with 4 Units of isoamylase (200 U/mL, Megazyme) and 55

µL sodium acetate buffer (1M, pH 4.0). Debranching by isoamylase was terminated by boiling the sample for 20 min. Freeze dried de-branched samples were suspended and vortexed in 200 µL of Dimethyl Sulfoxide, DMSO (99% v/v). Sample was then centrifuged at 15,000 g for 10 min. An aliquot (40 µL) of supernatant was injected into PL gel 5µM MiniMix-C guard column attached to PL gel MiniMix 250 x 4.6 mm ID column (Polymer Laboratories, Inc Amherst, MA) to separate amylose and amylopectin using high performance liquid chomatography system (Waters 600 Controller, Waters 610 Fluid Unit, Waters 717 plus Autosampler, Waters 410 Differential Refractometer, Waters Corporation, Milford, MA). DMSO (99% v/v) with lithium bromide 4.4% (w/v) was used as eluent at a flow rate of 0.2 mL/min with similar time interval of 30 min for injection and delay. Data was collected and analyzed using Empower 1154 Chomatography software (Waters Corporation, Milford, MA). Percent amylose was obtained by integrating peak area under amylose curve.

3.3.10 Starch morphology by atomic force microscopy (AFM)

Starch extracted from barley grains, as described above was suspended in water (1 mg/mL) and gelatinized (95 ^{o}C) and stored in a rapid visco analyzer (RVA) until deposition on mica. Sample temperature was reduced to 70 ^{o}C until 100 µl gelatinized starch (30 µg/mL) was deposited onto pre-heated freshly cleaved mica as an aerosol spray with nitrogen gas (Maley et al., 2010).

AFM images were taken using a PicoSPM instrument (Molecular Imaging, Tempe, AZ) which operates in intermittent contact mode. The force constant on the silicon cantilever (Nanoscience Instrument, Tempe, AZ), the resonant frequency and the curvature radius for AFM imaging were 48 N m^{-1}, 190 kHz and < 10 nm respectively. The ratio of set-point oscillation amplitude to free air oscillation amplitude was 0.75:0.85 while resonance amplitudes ranged from 0.4 to 1.0 V (Maley et al., 2010). The instrument was under ambient conditions and mounted in a vibration isolation system with a scan rate 1-1.5 Hz (512 pixels per line). Analysis of images and measurements were done using SPIP V5.0.5 software (Image Metrology, Denmark).

3.3.11 Amylopectin chain length distribution analysis

Amylopectin chain length distribution was determined by fluorophore-assisted capillary electrophoresis (FACE) (O'Shea et al., 1998) using the Proteome Lab PA800 (Beckman Coulter,

Fulerton, CA) equipped with a 488 nm laser module. Twenty milligrams of purified defatted starch samples in a microfuge tube (2 mL) were suspended in distilled water (750 µL) followed by the addition of 50 µL NaOH (2M). Samples were mixed vigorously and boiled for 5 min. Heated starches were allowed to cool at room temperature and neutralized with glacial acetic acid (32 µL). Sodium acetate buffer (1M, 100 µL) and distilled water (1 mL) were added to gelatinized starches. Gelatinized starches were debranched (37 °C for 2 h) with 10U of isoamylase (1000 U/mL) followed by boiling (10 min) and centrifugation (3000 g for 10 min). The supernatant was deionized by filtration though an ion exchange resin (20-50 mesh) in a microfuge tube. After deionization, 50 µL aliquot was dried for 30 min under vacuum (SPD SpeedVac, Thermo Electron Corporation, Milford, MA). De-branched chains were fluorescent labeled with 8-aminopyrene 1,2,6-trisulfonate (APTS) by overnight incubation of reaction mixture at 37 °C. The N-CHO (PVA) capillary with pre-burned window (50µm ID and 50.2 cm total length) was used for separation of debranched samples. Maltose was used as an internal standard. Samples (stored at 10 °C) were injected at 0.5 psia for 3 sec and separated at constant voltage of 30 kV for 30 min. Data was recorded and analyzed using 32-Karat software (Beckman Coulter). The degree of polymerization (dp) was assigned to peaks based on relative migration time of maltose used as an internal standard.

3.3.12 *In vitro* kinetics of starch enzymatic digestion

Barley pure starch and meal samples were enzymatically hydrolyzed *in vitro* for kinetic analysis (Englyst et al., 1992). Meal and pure starch (100 mg, in triplicates) samples were incubated with constant agitation in a 4 mL solution of pancreatic α-amylase (10 mg/mL) plus amyloglucosidase (3 U/mL) in sodium maleate buffer (0.1 M, pH 6.0). For kinetic analysis of starch hydrolysis, separate reaction mixtures were incubated for 30, 60, 120, 240 and 480 min incubation. The treatment without incubation was taken as 0 min of control. Enzyme treatment was terminated with 4 mL of ethanol (99%, v/v). After amylolysis termination, the reaction mixture was centrifuged (3000 g, 15 min) and the residue (isolated RS) was washed twice with ethanol (50% v/v). The final residue after centrifugation at 1500 g followed by drying was dispersed in 2 mL of potassium hydroxide (2M) and incubated in an ice water bath for 20 min with constant shaking. It was further incubated with amyloglucosidase (3300 U/mL) in 8 mL sodium acetate buffer (1.2 M, pH 3.8). Starch content (hydrolyzed and un-hydrolyzed) at the end

of each treatment was determined enzymatically by GOPOD kit method (AACC method 76-13). Hydrolyzed starch was determined as 'mg of glucose x 0.9'. Rate of starch digested (hydrolyzed) was expressed as percentage of total starch (TS) at the end of each interval.

3.3.13. Statistical analysis

All determinations were done in triplicate. The analysis of variance (ANOVA) of means was performed with SPSS univariate analysis (version10). Multiple means comparisons were determined with the Duncan's multiple range test at $p < 0.05$ confidence level. Cluster dendrogram analysis based on average linkage was performed using Minitab software (Version15) (Minitab, Inc. Pensylvania).

3.4 Results

3.4.1 Thousand grain weight (TGW)

TGW varied between genotypes. It was significantly reduced ($p < 0.05$) in atypical amylose genotypes (increased amylose or waxy) in comparison to normal amylose genotype (46.2 g, CDC McGwire). TGW was more severely affected in the increased amylose genotypes (range 32.4-37.4 g) than the waxy genotypes (range 34.1-45.1 g) (Table 3.1).

3.4.2 Protein concentration

Protein concentration differed among genotypes. The waxy genotype CDC Fibar recorded highest percent protein (16.6%) (Table 3.1). The values for the increased amylose genotypes ranged from 14.5 to 15%. A strong negative ($r = -0.80$; $p < 0.01$) correlation was observed between protein and total starch concentration.

3.4.3 Lipid concentration

Lipid concentration ranged from 3.0 to 4.4%, and varied significantly ($p < 0.05$) between genotypes (Table 3.1). Lipid concentration in the waxy (3.4-4.4%) and the increased amylose (3.2-3.4%) genotypes was comparatively greater than the normal starch genotype (3.0%). The waxy and increased amylose genotypes showed relatively higher concentration of lipids and dietary fiber.

Table 3.1. Carbohydrate and non carbohydrate contents from ten selected barley genotypes

Genotype	Starch Phenotype	Starch (%)	Amylose (%)	Protein (%)	TGW (g)	β-glucan (%)		TDF (%)	Crude Lipid (%)
						Calcofluor	Megazyme		
CDC McGwire	Normal	72.2 ± 0.6^{g}	25.8 ± 0.7^{d}	13.5 ± 0.0^{a}	46.2±0.7^{d}	5.8 ± 0.4^{a}	5.1 ± 0.4^{a}	11.8 ± 1.0^{a}	3.04 ± 0.4^{a}
SH99250	Increased amylose	61.1 ± 2.2^{bc}	38.5 ± 0.3^{e}	14.5 ± 0.1^{c}	37.4±0.3^{c}	11.3 ± 1.7^{d}	7.9 ± 0.6^{d}	17.4 ± 0.7^{e}	3.4 ± 0.2^{ab}
SH99073		59.7 ± 0.6^{ab}	40.8 ± 1.1^{f}	14.8 ± 0.0^{d}	32.4±0.4^{a}	9.7 ± 0.5^{bc}	8.5 ± 0.3^{de}	18.2 ±0.6^{e}	3.3 ± 0.2^{ab}
SB94893		61.9 ± 0.2^{c}	38.0 ± 0.1^{e}	15.0 ± 0.0^{e}	35.4±2.0^{b}	8.9 ± 0.3^{bc}	7.9 ± 0.1^{d}	16.0 ±0.7^{d}	3.2 ± 0.1^{a}
CDC Candle	Waxy	68.5 ± 0.9^{f}	4.5 ± 0.4^{c}	13.6 ± 0.0^{a}	45.1±0.1^{d}	6.8 ± 0.2^{a}	6.9 ± 0.1^{b}	13.6 ±0.5^{b}	3.4 ± 0.1^{ab}
CDC Rattan		65.6 ± 1.1^{de}	4.5 ± 0.1^{c}	15.0 ± 0.2^{e}	34.1±0.3^{b}	8.6 ± 0.1^{b}	7.4 ± 0.1^{c}	14.2 ± 0.8^{bc}	4.2 ± 0.6^{cd}
Waxy Betzes		66.5 ± 0.7^{e}	3.9 ± 0.1^{c}	14.2 ± 0.1^{b}	35.1±0.4^{b}	8.7± 0.4^{bc}	7.1 ± 0.0^{bc}	15.13±1.0^{cd}	3.4±0.5^{ab}
SB94912		61.0 ± 0.7^{bc}	1.6 ± 0.5^{b}	15.2 ± 0.1^{f}	36.8±0.2^{c}	9.8 ± 0.2^{c}	8.2 ± 0.2^{de}	15.0 ±0.2^{cd}	3.8 ± 0.2^{bc}
CDC Alamo		64.2 ± 0.2^{d}	nd^{a}	15.0 ± 0.0^{e}	37.8±1.0^{c}	8.7 ± 0.4^{bc}	7.3 ± 0.1^{bc}	13.7 ±0.4^{b}	4.4 ± 0.3^{d}
CDC Fibar		58.1 ± 1.8^{a}	nd^{a}	16.6 ± 0.2^{g}	37.1±0.2^{c}	13.3 ± 0.1^{f}	9.7 ± 0.4^{f}	17.5 ± 0.6^{e}	3.6 ± 0.1^{ab}

(i) Data reported on dry basis (db) and represent means of three biological replications and two independent observations for each replicate ± standard deviation (SD). (ii) Mean values within a column followed by different superscripts are significantly different ($p<0.05$).

3.4.4 Total Dietary Fiber (TDF) concentration

Total dietary fiber concentration ranged from 11.8 to 18.2% and differed significantly (*$p < 0.05$)* among genotypes (Table 3.1). CDC McGwire (normal starch) had the lowest TDF at 11.8%, whereas SH99073, an increased amylose genotype, had the highest (18.2%) TDF (Table 3.1). CDC Candle (13.6%) and CDC Alamo (13.7%) had the lowest TDF among the waxy genotypes with no significant ($p < 0.05$) difference between them. CDC Fibar (17.5%) had the highest TDF among the waxy genotypes. Increased amylose genotypes had significantly ($p < 0.05$) higher dietary fiber concentration compared to that of either normal or waxy genotypes. Among increased amylose genotypes SH99073 had the highest (18.2%) TDF followed by SH99250 (17.4%) and SB94893 (16%). Variation in dietary fiber concentration among waxy genotypes was 3.9%, whereas 2.2% was observed for the increased amylose genotypes. These results concur with an earlier observation that dietary fiber concentrations are higher in increased amylose genotypes (Bjork et al., 1990).

3.4.5 Beta-glucan concentration

There were significant *($p < 0.05$)* differences in β-glucan concentration among genotypes and between methods used to determine β-glucan concentration. However, the ranking of genotypes for β-glucan concentration was same for both methods. The calcofluor method gave slightly higher values (0.7 to 3.4%) than the Megazyme method (Table 3.1). The difference observed could be due to interference from cellulosic components such as xylans (Wood and Fulcher, 1978; Jørgensen and Aastrup, 1988). Values for the calcofluor method ranged from 5.8% to 13.3% whereas Megazyme method values ranged from 5.1 to 9.7%. The normal starch genotype, CDC McGwire recorded the lowest β-glucan concentration in both methods at 5.8% and 5.1% for the calcofluor and megazyme methods respectively. The waxy and increased amylose genotypes recorded significantly higher β-glucan concentration compared to that of the normal starch genotype. CDC Fibar had significantly higher β-glucan concentration either from the Calcofuor (13.3%) or Megazyme (9.7%) method but lower starch concentration and non detectable amylose (Table 3.1). Among waxy genotypes, CDC Candle had the lowest β-glucan concentration using both methods and varied by 0.1%. CDC Alamo had 8.7% and 7.3% β-glucan from the Calcofluor and Megazyme methods respectively. β-glucan concentration values for

CDC Rattan and SB94912 varied by 1.2% and 1.6% respectively, using Megazyme and Calcoflour methods. Values from the Calcofluor method for the increased amylose genotypes were 11.3%, 9.7% and 8.9% for SH99250, SH99073 and SB94893 respectively. However, corresponding values from the Megazyme method were significantly lower (7.9%, 8.5% and 7.9% respectively).

3.4.6 Total starch concentration

The total starch concentration of the ten genotypes varied significantly ($p < 0.05$) and ranged from 58.1% to 72.2%. Similar to TGW, total starch concentration was significantly reduced in atypical amylose genotypes (Table 3.1).

3.4.7 Starch granule size

Starch granule diameter ranged from 1.3 - 45.7µm (Figure 3.1a). Increased amylose genotypes (SH99250, SH99073 and SB94893) showed skewed starch granule distribution compared to that of CDC McGwire and the waxy genotypes. Mean surface diameter of the starch granules decreased with altered amylose concentrations for all genotypes versus CDC McGwire (16.3 µm). For waxy starch genotypes mean starch granule size ranged from 12.9-15.9 µm whereas for increased amylose genotypes, it was lower, ranging from 8.2-9.5 µm. Division of starch granules in the diameter range of > 5 µm (C-type), 5-15 µm (B-type) and > 15 µm (A-type) showed significant reduction ($p < 0.05$) in volume percentage of A-type granules in all genotypes in comparison to CDC McGwire (Figure 3.1b). Increased amylose genotypes showed a dramatic increase in B-type starch granules (68 to 78%) with a corresponding significant reduction ($p < 0.05$) in A-type starch granules (12 to 19%) in comparison to that of waxy genotypes.

3.4.8 Amylose concentration

Six genotypes, CDC Rattan, CDC Fibar, CDC Candle, Waxy Betzes, SB94912 and CDC Alamo had less than 5% amylose and hence were classified as either waxy or near waxy genotype (Table 3.1). CDC McGwire, a normal starch genotype had 25.8% amylose while three barley genotypes (SH99250, SH99073 and SB94893) had amylose concentration more than ~40% and were therefore classified as increased amylose genotypes.

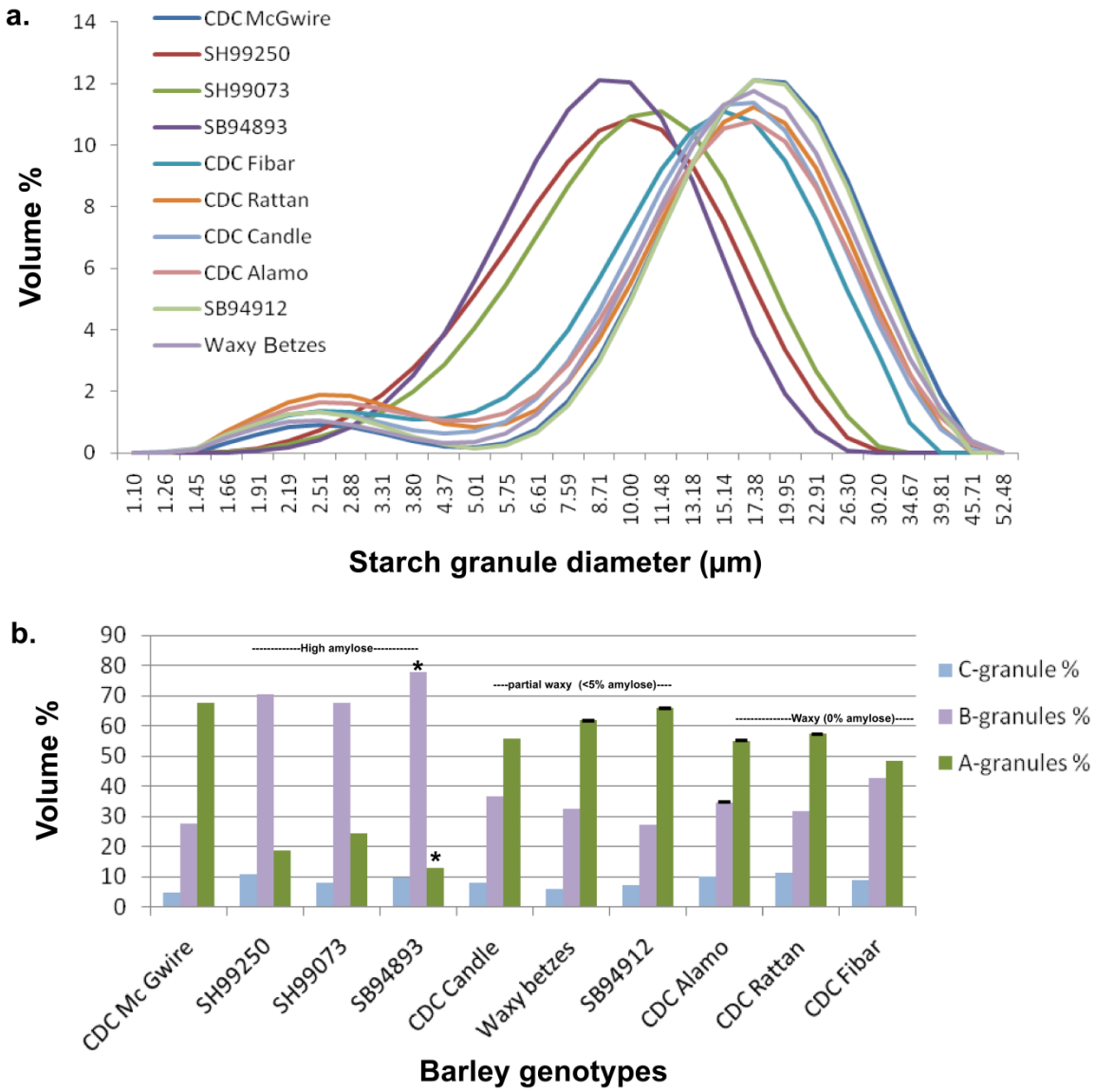


Figure 3.1 Starch granule distribution
A normal starch (CDC McGwire), increased amylose starch (SH99250, SH99073, SH94893) and waxy starch (CDC Fibar, CDC Rattan, CDC Candle, CDC Alamo, SB94912 and Waxy Betzes) genotypes of barley (a); Effect of amylose content on C-type (< 5µm), B-type (5 - 15µm and A-type (>15 µm) diameter range of starch granules (b). (* indicate maximal increase in B-type and decrease in A-type starch granules volume percent as compared to normal starch genotype CDC McGwire)

3.4.9 Starch morphology

A previously developed spray deposition method (Maley et al., 2010), applying hot starch suspensions (95 ^{o}C), deposited starch evenly on freshly cleaved mica to avoid multi-layers and aggregation observed in the drop deposition method. Images for a waxy starch genotype (undetectable amylose) showed large biopolymer fibrils with an average height of 1.9 nm. Lengths run into several µm long as single fibrils or bundled together in an intertwining structure (Figure 3.2a). A near waxy genotype (< 5% amylose) showed small biopolymer strands with an average height of 0.7 nm and nm-scaled length (Figure 3.2b), indicating these are amylose. The height and length of large biopolymer fibrils were similar to those observed in Figure 3.2a. The long biopolymer fibrils were considered to be amylopectin in starch granules that did not completely gelatinize during starch preparation process.

AFM for normal starch (~25% amylose) and increased amylose starch (> 38% amylose) genotypes showed large biopolymer fibrils with average height and length of 2.6 to 2.9 nm and 15 µm respectively (Figure 3.2c, d). These were also observed as bundled fibrils in a continuous network and were considered to be amylopectin. Significant ($P < 0.05$) differences were observed in large biopolymer fibril lengths from waxy genotypes (1.9 nm) and genotypes with ~25% or increased amylose concentration (2.6 to 2.9 nm). Sections containing smaller fibrils (amylose fibrils) from normal and increased amylose genotypes were magnified and had nm-length fibrils with average height ranging from 0.7 to 0.8 nm, as also observed from amylose in near waxy (< 5% amylose) genotypes (Figure 3.2 c,d). McIntire and Brant (1999) estimated a single amylose chain height as 0.54 nm by using non-contact AFM.

Amylose biopolymer fibril chains, produced using an aerosol spray method, were well-spaced and therefore allowed contour length estimation. Contour lengths were 130 ± 60, 202 ± 141 and 214 ± 108 for near waxy, normal and increased amylose starch genotypes, respectively. It has been proposed in earlier reports that amylose (a fairly linear glucose polymer) adopts a shape with six sugar residues in each turn (V-shape) and 1.32 Å rise per residue with a linear mass density of 1220 Da nm^{-1} (Winger et al., 2009; French and Zaslow, 1972). On the basis of this, the degree of polymerization, the polydispersity index (distribution of molecular mass), weight average molecular weight (M_w) and the number average molecular weight (M_n) were estimated. Waxy starch had a very low polydispersity index and DP, while normal starch and increased

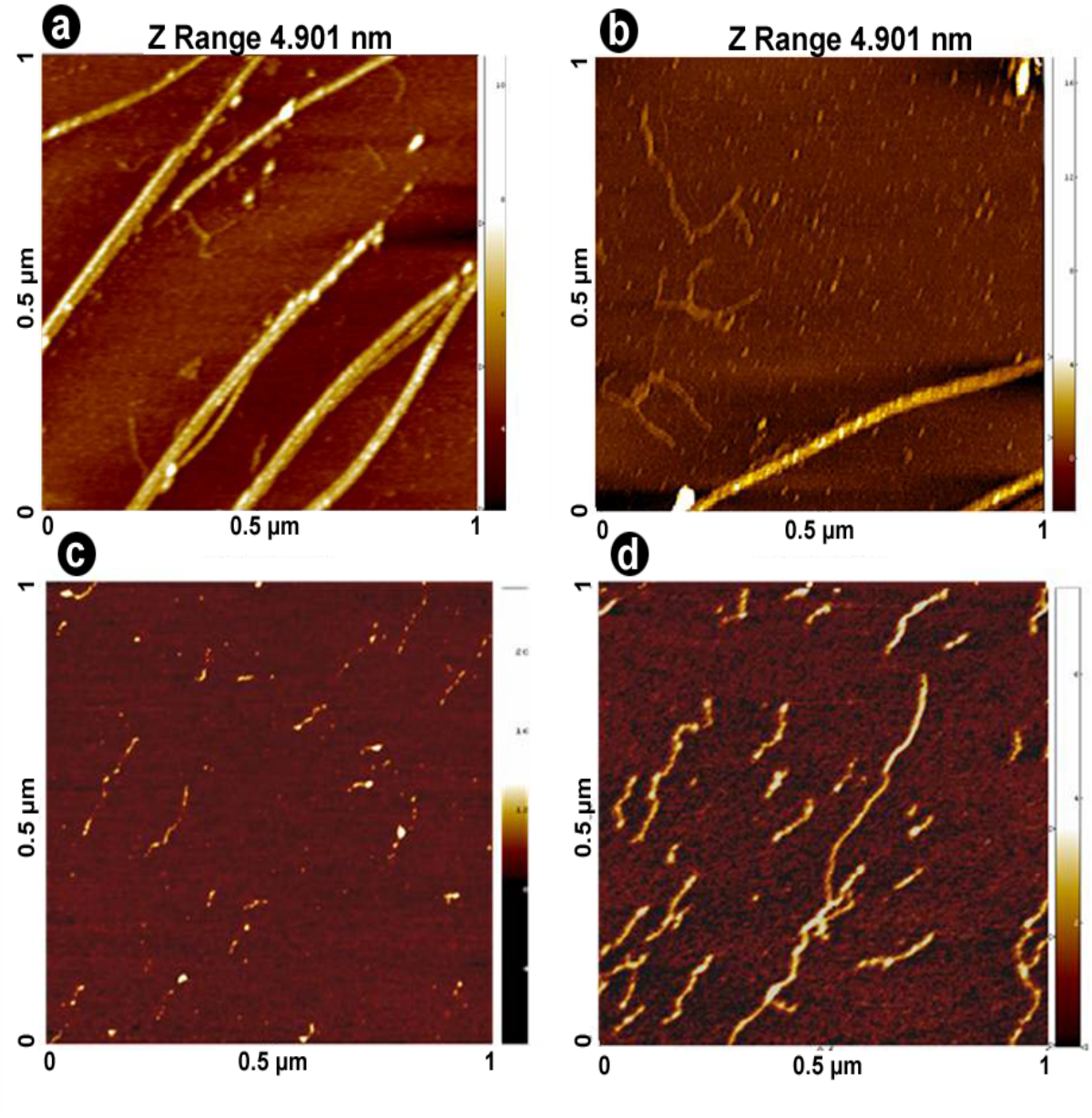


Figure 3.2 Atomic force microscopy (AFM) images from barley genotypes varying in amylose concentration accumulated in the endosperm.
Biopolymer fibrils of amylose in waxy (a); near waxy (< 5% amylose) (b); normal (~25% amylose) (c) and increased amylose genotypes (d).

amylose starch showed no significant difference in polydispersity index, but increased amylose starch had higher DP than normal starch (Table 3.2).

Table 3.2. Biopolymer fibrils properties of starch amylose from normal, increased amylose and waxy hulless barley genotypes

Barley Phenotype	Amylose (%)	Height (nm)	Contour Length (nm)	M_n ($x10^5$Da)	M_w ($x10^5$ Da)	Polydispersity Index (M_w/M_n)	DP
Normal	25.8	0.8	202 ± 141^b	2.46	3.64	1.48^b	1530^b
Increased amylose	>38	0.8	214 ± 108^c	2.62	3.28	1.25^b	1620^c
Waxy	<5.0	0.7	130 ± 60^a	1.59	1.92	0.33^a	985^a

Values of amylose properties obtained from 36 or more different biopolymer chains. Means within the same column with different superscripts are significantly different ($p < 0.05$)

3.4.10 Amylopectin chain length distribution (CLD)

Depending on the changing slopes, FACE characterized chain length distribution (CLD) curve was divided into four fractions DP 6-11(F-I), DP 12-18 (F-II), DP 19-36 (F-III) and DP >37 (F-IV) as indicated in CLD graph from CDC McGwire (Figure 3.3). More than 60% of total DP area was occupied by DP 6-18 fractions. CDC Rattan and CDC Fibar were comparatively enriched in F-I, DP 6-11 fraction than other genotypes. SH99250 was unique, having significantly higher ($p < 0.05$) F-II (DP 19-36) fraction and reduced F-I (DP 6-11) fraction (Table 3.3). CLD was significantly ($p < 0.05$) correlated with protein and starch concentrations. Amylose concentration affected F-I and F-III fractions of amylopectin in a significant but opposite manner. Significant increase in F-III chains ($r = 0.37$, $p < 0.05$) of amylopectin was observed with increasing amylose concentration in selected genotypes.

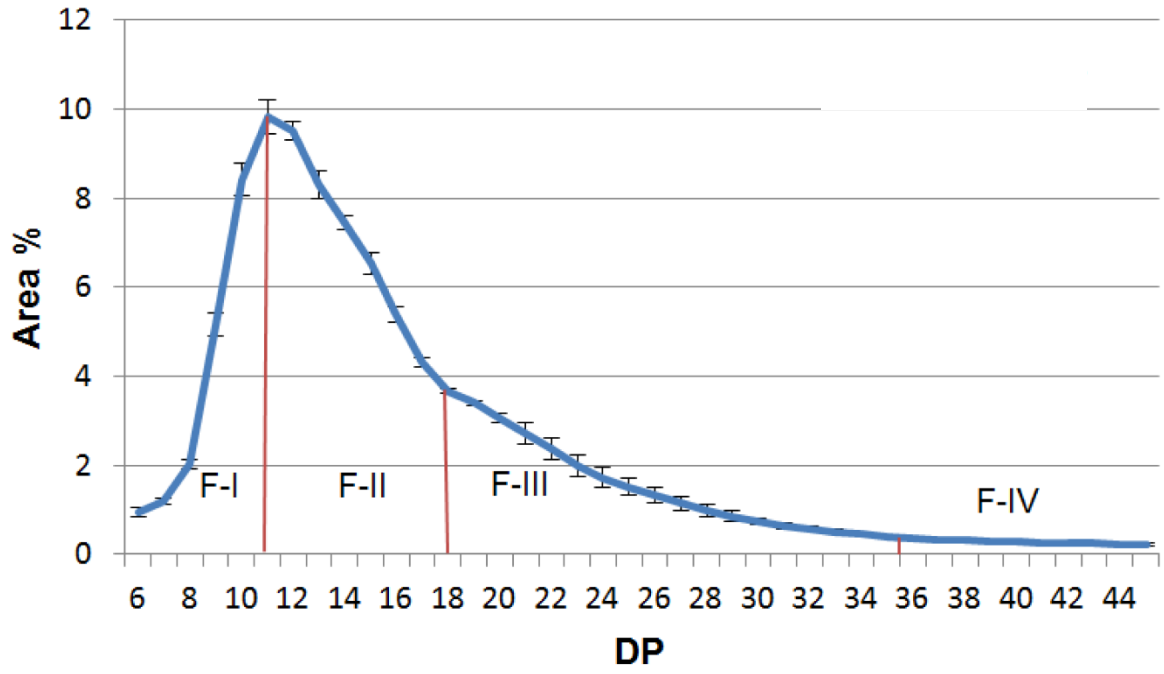


Figure 3.3 Amylopectin unit chains divided into four fractions
F-I (DP 6 - 11), F-II (DP 12 - 18), F-III (DP 19 - 36) and F-IV (> DP 37), based on changing slopes as depicted in normal starch barley genotype CDC McGwire.

3.4.11 Starch enzymatic hydrolysis

On the basis of the time required for starch enzymatic hydrolysis, starch can be divided into rapidly digestible (RDS), slowly digestible (SDS) and resistant starch (RS). RDS can be defined as the starch fraction hydrolyzed within 20-30 min of incubation with RS the fraction remaining un-hydrolyzed even after 180 min of incubation. The difference between the two types is termed SDS. The barley starch enzymatic hydrolysis rate was analyzed for an 8 h incubation period, however more than 80% starch was hydrolyzed within 4 h in all samples (both meal and pure starch) (Appendices 3.1 and 3.2). RDS values in pure starch samples ranged from 32.7-82.1% (Table 3.4). As expected, the RDS values in pure starch samples were higher than in the meal samples for the same genotype. RDS followed the order: waxy > normal > increased amylose. SDS content varied significantly among genotypes and between sample types. SDS values in meal samples ranged from 42.3-48.2%, whereas values for pure starch ranged from 17.9-49.6%. Differences in SDS content were higher in pure starch than in meal samples (Table 3.4). SDS is the most important dietary starch and values in the pure starch samples followed the order increased amylose > normal > waxy genotype.

Table 3.3. Amylopectin chain length distribution (CLD) in ten hulless barley genotypes with varied grain starch amylose concentration

Genotype	Distribution (%)				Average DP*
	DP 6-11 F- I	DP 12-18 F- II	DP 19-36 F- III	DP 37-45 F- IV	
CDC McGwire	27.55±1.09^{d}	45.13±1.27^{b}	24.86±2.11^{ab}	2.46±0.26^{a}	16.18±0.35
SH99250	18.49±1.75^{a}	43.36±0.77^{b}	34.73±2.38^{e}	3.43±0.19^{a}	18.07±0.40
SH99073	23.10±2.86^{bc}	43.32±4.05^{b}	30.42±2.45^{cd}	3.15±0.47^{a}	17.26±0.32
SH94893	25.39±0.87^{cd}	43.64±1.96^{b}	28.32±0.51^{bcd}	2.63±0.58^{a}	16.72±0.21
CDC Candle	23.37±2.10^{bc}	43.36±1.69^{b}	29.36±2.65^{cd}	3.92±0.91^{a}	17.32±0.63
Waxy Betzes	22.08±1.70^{b}	43.86±1.40^{b}	30.37±1.88^{cd}	3.70±0.56^{a}	17.49±0.44
SB94912	21.67±1.54^{b}	42.70±0.67^{b}	31.47±1.16^{de}	4.15±0.48^{a}	17.76±0.38
CDC Alamo	26.93±0.49^{d}	41.82±0.07^{ab}	27.71±2.95^{bc}	3.54±2.67^{a}	16.95±0.52
CDC Rattan	30.73±2.86^{e}	43.95±2.89^{b}	22.84±0.60^{a}	2.48±0.51^{a}	15.76±0.25
CDC Fibar	32.23±1.18^{e}	38.87±0.07^{a}	24.86±0.74^{ab}	4.04±0.54^{a}	16.36±0.30

(i) *Average DP=$\sum$ (DP_n x peak area)/$\sum$ $(\text{peak area})_n$
(ii) Means within the same columns with different superscripts are significantly different ($p < 0.05$)

In both meal and pure starch samples, RS concentration followed the order increased amylose > normal > waxy genotypes. RS concentrations were significantly higher in meal than pure starch samples for all genotypes. Endogenous amylase inhibitors present in barley meal may be responsible for the observed difference in meal and pure starch hydrolysis (Weselake et al., 1985). In meal samples RS concentration for increased amylose genotypes ranged from 22.4 to 26.5%, whereas it ranged from 15.4 to 28.1% in pure starch samples. However increased amylose genotype SH99250 was an exception as RS in meal was 1.5% lower than pure starch sample.

Table 3.4. Hydrolytic analysis of meal and pure starch samples from ten selected hulless barley genotypes

Genotype	RSM	RSPS	RDSM	RDSPS	SDSM	SDSPS
CDC McGwire	7.3 ± 0.7^{de}	8.3 ± 0.3^{e}	50.7 ± 1.0^{ef}	41.8 ± 0.6^{c}	42.3 ± 0.8^{a}	49.6 ± 0.6^{g}
SH99250	26.5 ± 0.8^{g}	28.1 ± 0.5^{h}	27.6 ± 1.0^{b}	32.7 ± 0.5^{a}	46.0 ± 0.3^{def}	39.2 ± 1.0^{f}
SH99073	26.3 ± 0.3^{g}	24.3 ± 0.2^{g}	25.4 ± 0.8^{a}	35.3 ± 0.6^{b}	48.2 ± 0.9^{g}	40.5 ± 0.8^{f}
SB94893	22.4 ± 0.5^{f}	15.4 ± 1.7^{f}	33.1 ± 0.9^{c}	52.2 ± 0.4^{d}	44.2 ± 0.3^{bc}	32.3 ± 1.0^{c}
CDC Candle	4.4 ± 0.3^{a}	3.0 ± 0.1^{d}	51.8 ± 0.9^{f}	60.3 ± 0.5^{e}	43.9 ± 1.0^{abc}	36.7 ± 1.0^{e}
Waxy Betzes	5.7 ± 0.3^{bc}	1.2 ± 0.4^{bc}	51.2 ± 0.9^{f}	66.1 ± 0.2^{g}	43.2 ± 1.0^{ab}	32.8 ± 0.9^{cd}
SB94912	6.5 ± 0.5^{cd}	1.8 ± 0.7^{c}	46.8 ± 0.8^{d}	65.3 ± 0.3^{fg}	46.8 ± 1.0^{efg}	32.2 ± 1.4^{c}
CDC Alamo	5.6 ± 0.3^{b}	0.1 ± 1.1^{a}	49.4 ± 0.6^{e}	82.1 ± 0.4^{i}	45.2 ± 1.0^{cde}	17.9 ± 0.3^{a}
CDC Rattan	5.0 ± 0.1^{ab}	1.4 ± 0.3^{c}	47.9 ± 0.3^{d}	64.6 ± 0.9^{f}	47.1 ± 1.0^{fg}	34.3 ± 0.9^{d}
CDC Fibar	7.9 ± 0.7^{e}	0.5 ± 0.5^{ab}	47.8 ± 1.0^{d}	76.9 ± 1.0^{h}	44.4 ± 1.0^{bcd}	22.5 ± 0.9^{b}

(i) Hydrolysis data based on average of thee replicates with two observations for each replicate ± standard deviation (SD). (ii) Mean values in a column followed by the same letter(s) are not significantly different ($p < 0.05$).

3.5 Discussion

A balanced concentration of protein, fat, carbohydrate, vitamins and minerals provided by cereal grains is essential for human development and healthy living. The correlation between increased amylose and resistant starch has increased its utility to develop diet-based solutions for disease prevention and healthy living. It has been observed that, similar to fiber, RS also plays a useful role in preventive health care in diseases like colon cancer, diabetes, obesity, osteoporosis and cardiovascular diseases (Sharma et al., 2008). In comparison to amylopectin, amylose contributes towards low glycemic index and also promotes bowel health, thereby, reducing colorectal cancer risk. Analysis of the grain constituents and starch characteristics of ten hulless barley genotypes with varied carbohydrate composition revealed a high correlation between grain constituents and starch enzymatic digestibility indices. A negative correlation between amylose and starch enzymatic digestibility was observed. The normal starch genotype (CDC McGwire) had the highest starch concentration (72%) as compared to increased amylose, waxy or near waxy genotypes, (range 58-68%). Total starch concentration was an indicator of higher grain weight as shown by significant positive correlation ($r = 0.70$, $p < 0.05$) between TGW and total starch concentration (Table 3.5).

Table 3.5. Correlation analysis between barley grain components affecting starch enzymatic hydrolysis

	Starch	Amylose	Protein	TGW	F-I	F-II	F-III	F-IV	C-granule	B-granule	A-granule	RSM	RSPS	RDSM	RDSPS	SDSM	SDSPS	BGC	BGM	TDF	Lipid
Starch	1																				
Amylose	-0.15	1																			
Protein	-0.80**	-0.25	1																		
TGW	0.70*	-0.11	-0.56**	1																	
F-I	0.1	-0.40*	0.45*	0.02	1																
F-II	0.46*	0.29	-0.60**	0.17	-0.36*	1															
F-III	-0.30	0.37*	-0.2	-0.12	-0.89**	0.10	1														
F-IV	-0.24	-0.29	0.16	0.04	-0.18	-0.44*	0.2	1													
C-granule	-0.48**	0.02	0.44*	-0.44*	0.12	-0.23	0.01	-0.05	1												
B-granule	-0.52**	0.83**	0.13	-0.40*	-0.31	0.01	0.39*	-0.14	0.42*	1											
A-granule	0.55**	-0.79**	-0.17	0.43*	0.28	0.02	-0.37*	0.14	-0.51**	-0.99**	1										
RSM	-0.50**	0.91**	0.05	-0.40*	-0.43*	0.09	0.48**	-0.15	0.30	0.94**	-0.93**	1									
RSPS	-0.31	0.94**	-0.17	-0.22	-0.51**	0.21	0.51**	-0.18	0.21	0.84**	-0.82**	0.96**	1								
RDSM	0.56**	-0.86**	-0.12	0.49**	0.41*	-0.07	-0.47**	0.13	-0.37*	-0.90**	0.91**	-0.98**	-0.93**	1							
RDSPS	-0.06	-0.89**	0.46*	-0.06	0.48**	-0.43*	-0.37*	0.27	0.12	-0.58**	0.54**	-0.75**	-0.89**	0.71**	1						
SDSM	-0.55**	0.13	0.36	-0.62**	-0.10	-0.06	0.15	0.02	0.50**	0.26	-0.30	0.36*	0.32	-0.50**	-0.15	1					
SDSPS	0.47**	0.57**	-0.67**	0.35	-0.32	0.56**	0.11	-0.31	-0.46*	0.11	-0.05	0.29	0.50**	-0.25	-0.84**	-0.09	1				
BGC	-0.90**	-0.01	0.82**	-0.54**	0.07	-0.52**	0.15	0.26	0.45*	0.36	-0.38*	0.32	0.18	-0.37*	0.14	0.33	-0.48**	1			
BGM	-0.91**	-0.05	0.84**	-0.64**	0.10	-0.55**	0.13	0.3	0.48**	0.40*	-0.43*	0.31	0.11	-0.39*	0.22	0.47**	-0.55**	0.87**	1		
TDF	-0.82**	0.40*	0.53**	-0.66**	-0.23	-0.27	0.38*	0.17	0.36	0.68*	-0.69**	0.68**	0.55**	-0.71**	-0.25	0.45*	-0.16	0.77**	0.80**	1	
Lipid	-0.12	-0.58**	0.35	-0.25	0.27	-0.14	-0.29	0.22	0.48**	-0.36*	0.30	-0.40*	-0.46*	0.29	0.58**	0.30	-0.58**	0.16	0.17	-0.1	1

*indicate significance at $p<0.05$
** indicate significant at $p<0.01$

Starch granule size showed bimodal distribution in normal and waxy genotypes. For increased amylose genotypes, starch granule size distribution appeared unimodal (Figure 3.1). In CDC McGwire and waxy genotypes, the highest volume percentage was occupied by starch granules with diameter between 15-17 µm. Increased amylose genotypes (SH99250, SH99073 and SB94893) showed altered starch granule distribution with the highest volume percentage occupied by granules between 8 and 11 µm in diameter. Decrease in the mean starch granule diameter observed in the present study concurs with the results observed in high amylose "Glacier" barley (Banks et al., 1971). Amylose concentration was strongly correlated ($r = 0.83$, $p < 0.01$) with B-type starch granule (5-15µm) content, concurring with an earlier report (You and Izydorczyk, 2002) (Table 3.5). Starch hydrolysis studies showed significant positive correlation of RS with B-type starch granule number ($r = 0.84$, $p < 0.01$) and amylose concentration ($r = 0.94$, $p < 0.01$) while negative correlation with A-type starch granule number ($r = -0.82$, $p < 0.01$) (Table 3.5). Comparative analysis revealed that the medium sized B-type starch granule with increased amylose contributed most towards resistant starch formation.

Amylopectin structure and concentration contributes to starch uniformity, stability, texture and better freeze thaw abilities (Chibbar and Chakraborty, 2005). Amylopectin chain length distribution (CLD) and packing have been reported as an important characteristic of starch digestibility (Benmoussa et al., 2007). Amylopectin unit chains F-II (DP 12-18) were strongly correlated ($r = 0.56$, $p < 0.01$) with SDS content of pure starch (Table 3.5). This indicates a relationship between amylopectin unit chain length distribution and starch digestibility. However, no correlation was found in meal samples suggesting interference of non-starch components in starch digestibility. Increased amylose in barley genotypes affected CLD by influencing shorter chains F-I in negative, and longer or intermediate F-III chains of amylopectin in a positive manner. In fact F-III (DP 19 - 36) chains were synergistic with amylose concentration in RS formation for both meal and pure starch samples (Table 3.3).

Amylopectin biopolymers were common for all samples, however amylose concentration was the only factor separating starch types. Amylose is the principal component in the formation of RS Type 2 and 3 (Banks et al., 1971). For this reason we also estimated polymer properties of amylose which has unique features in different starch samples. Differences in contour lengths of biopolymer fibrils from normal, increased amylose and waxy (< 5% amylose) genotypes could

explain the packaging of amylose within the amorphous matrix of an amylopectin molecule. The waxy (undetectable or < 5% amylose) genotypes will have less compact structure compared to the normal and increased amylose starch genotypes.

This could be one of the reasons for ease of gelatinization and enzymatic hydrolysis of waxy starch in comparison with increased amylose starch. The polydispersity index was obtained from the ratio of M_w to M_n and showed significant differences between the genotypes (Table 3.2). The degree of polymerization (DP) was significantly higher in normal (1530) and increased amylose (1620) compared to that of waxy genotypes (985).

The kinetics of hydrolytic studies showed an initial rapid phase of RDS release followed by a slower phase of SDS release. The lowest starch digestion rate in all stages was observed for the increased amylose genotypes (SH99250, SH99073 and SB94893) whereas the highest was observed for waxy genotypes (Table 3.3). The AFM results for amylose morphology indicated higher polydispersity indices for normal (1.48) and increased amylose starch genotypes (1.25) compared to partially waxy starch genotype (0.33) (Table 3.2). This suggests there were too many molecules within a defined length in the first two genotypes as compared to the latter. The expected resultant effect on starch gelatinization and enzymatic hydrolysis would be faster rate in the latter than the former.

The observed effect corroborates the hydrolysis finding where rate of enzymatic starch hydrolysis was faster for waxy genotypes compared with that of genotypes with > 25% amylose. The energy required to gelatinize and completely hydrolyze starch into glucose will obviously be lower for waxy starch compared to that for either normal or increased amylose starch. This also makes waxy starch more susceptible to hydrolytic enzymes in comparison to starch granules with significant amylose concentration, which interferes with the access of hydrolytic enzymes to amorphous zone of starch granules.

Waxy or near waxy starch genotypes can be recommended for famine prone regions as they release glucose rapidly during digestion whereas increased amylose types could be recommended for diabetic diets due to associated slow release of glucose (low glycemic index). Total starch concentration was lower in waxy and increased amylose genotypes implying that the altered amylose to amylopectin ratio negatively affects starch concentration.

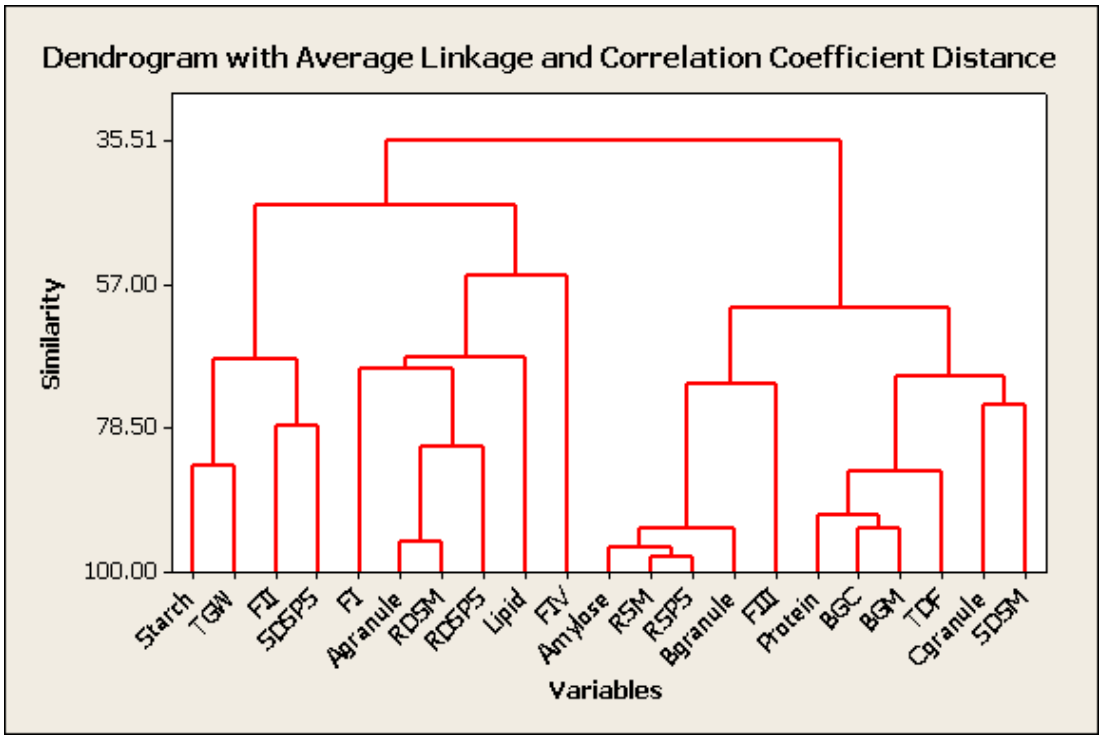


Figure 3.4 Average linkage dendrogram depicting physical relationship between different components of barley grain related to starch enzymatic hydrolysis.

Barley genotypes with atypical amylose had higher β-glucan concentration compared to normal starch genotypes, in agreement with earlier reports on altered amylose and its effect on starch and β-glucans (Izdorczyk et al., 2000). High β-glucan concentration in barley makes it more useful in human nutrition due to associated health benefits. Malting and feed barley (for monogastric animals) are selected for lower β-glucan concentration. β-glucan concentration in hulless barley ranged from 3.0% to 7.0% (Izdorczyk et al., 2000). This study indicates substantial improvement in β-glucan concentration especially with atypical amylose concentration. In the present study, β-glucans affected SDS in meal samples ($r = 0.47$, $p < 0.01$), decreasing RDS concentration by inhibiting starch hydrolysis.

Cluster dendrogram analysis (Figure 3.4) also revealed patterns of relationships between grain constituents and starch characteristics related to starch digestibility. The dendrogram analysis based on average linkage showed a right branch- left cluster group consisting of RSM, RSPS and amylose which is linked to B-type starch granule content and influenced maximally by the F-III

fraction of amylopectin. The right sub-group of the above right cluster exhibited strong correlation between protein, BGC and BGM having > 80% similarity with TDF and this set has an average > 55% similarity with both C-type starch granule and SDSM (strongly intra-correlated) pair and with the left sub-group containing amylose. The left branch cluster group has starch and TGW (> 80% intra-correlation) pair and F-II and SDSPS pair which depicts > 55% similarity level and forms the left sub-group of this cluster. The right sub-group of left branch cluster group exhibits strong correlation between A-granules and RDSM which is further influenced by RDSPS (> 80% similarity level) and F-I. This is linked with lipid at > 60% similarity level. This right sub-group also has F-IV at a 55% level of similarity with other variables of this right sub-group. Within the left branch, the two sub-groups have 40% similarity. This analysis revealed that variation in amylose concentration significantly influenced resistant starch content in meal and pure starch. These factors are further affected strongly by B-type starch granules (5-15 µm) and amylopectin F-III (19 - 36 DP) fraction (Figure 3.3). Amylopectin medium chains of DP 12-18 (F-II) influenced SDS in pure starch samples ($r= 0.56$, $p < 0.01$)

The major challenge in development of increased amylose genotypes is reduced grain size. The known increased amylose barley mutants Himalaya 292 (sex6 mutant) (65-70% amylose, *SsIIa* mutation*), Sbe IIa* and *b* mutant (> 65% amylose) and *Amo1* etc. are reported to have reduced starch content with increased fiber, lipid and phosphate (Morell et al., 2003; Borén et al., 2008; Regina et al., 2010). This indicates the need to adopt a breeding approach to preferably use a specific genotype should also consider overall yield and large grain size. In the present study TGW positively correlated with percent total starch but negatively with protein. Increase in protein could be the result of interference in carbon partitioning between protein and carbohydrates and hence decreased starch concentration as observed in altered amylose genotypes. The effect of atypical amylose on lipid concentration and its interaction with starch needs to be investigated.

This study indicates that barley cultivars with increased F-III chains of amylopectin and increased amylose could be selected for breeding lines with higher resistant starch content. The optimization of the data suggested that SH99250 with less decrease in grain weight and enriched DP 19-36 amylopectin in comparison to other increased amylose genotypes (SH99073, SH94893) may be a promising genotype for developing cultivars with increased amylose concentration. Recently an increased amylose genotype, CDC Hilose containing 40% amylose has been developed from a cross between SH99250 and CDC McGwire (http://www.inspection.gc.ca/).

CHAPTER 4

Gbss1 POLYMORPHISM IN BARLEY GENOTYPES WITH ALTERED AMYLOSE CONCENTRATION IN STARCH

4.1 Abstract

The accumulation of GBSSI within starch granules and structure of *GbssI* alleles were determined for nine barley genotypes producing amylose-free (undetectable), near waxy (1.6 - 4.5%), normal (25.8%) and (38.0-40.8%) increased amylose grain starches. As compared to normal starches, GBSSI accumulation was drastically reduced in starch granules of three near waxy genotypes, but only slightly reduced in two waxy starches and slightly elevated in three increased amylose starches. An alignment of *GbssI* sequences determined for the nine genotypes with published alleles distinguished three *GbssI* groups. One clearly distinguishable *GbssI* type, exclusive to three near waxy lines, showed a 403-bp deletion in region covering the TATA-box, non-translated exon 1 and part of intron 1. This mutation did not totally eliminate GBSSI or amylose production, presumably due to weak transcription from an alternate promoter. The near waxy genotype SB944912 with a lower amylose production (1.6%) than near waxy genotypes CDC Rattan and CDC Candle (4.5% amylose) could possibly be explained by a unique Q312H substitution within GBSSI that might have reduced enzyme activity. The GBSSI produced by waxy genotypes CDC Alamo and CDC Fibar showed a D287V change for CDC Alamo and a G513W change for CDC Fibar. Both amino acid alterations involved a conserved residue within domains proposed to be involved in glucan interaction. Thus, GBSSI inability to bind substrate or target was likely the cause for lack of amylose production in the waxy genotypes. Increased amylose genotypes SH99250, SH99073 and SB94893 carried several unique nucleotide changes within the second and fourth *GbssI* introns and one unique amino acid substitution, A250T, was noted for GBSSI produced by SB94893. None of the observed *GbssI* sequence differences could be associated with increased amylose phenotype for all three increased amylose genotypes. Genetic markers for DNA sequences affecting *GbssI* expression or GBSSI activity were developed and could be used to monitor various *GbssI* alleles in future studies.

4.2 Introduction

The composition and structure of starch produced in cereal endosperm are important grain quality traits determining the suitability of grain for different applications. Grain with varied amylose concentration is of particular interest for starch industries providing products for niche markets. As compared to normal starches, low or amylose-free (waxy) starches provide improved freeze-thaw stability and they gelatinize easily to form non-gelling pastes suitable for food thickening and stabilization (Jobling, 2004). High-amylose starches have a high gelling strength and are used for production of resistant starches, formation of films and as adhesives in the board and paper industries. Some of the desired starch structures can be attained by chemical treatments (Albertsson and Karlsson, 1995; Fang et al., 2004); however, these methods are expensive and use chemicals with negative effects on the environment. To develop plants with desired starch composition and structure for various food and industrial applications, it is important to identify genes that affect starch biosynthesis in desired direction. Genetic markers for these key alleles can thus be used to accelerate breeding of lines with novel starch properties.

The granule-bound starch synthase (GBSS1) is the sole enzyme responsible for amylose biosynthesis in plants (Shure et al., 1983). Besides producing amylose, the enzyme has also a role in extending amylopectin chains (Denyer et al., 1999a; 1999b). GBSSI is a member of the large glycosyltransferase family, which catalyzes monosaccharide transfers, usually a nucleotide diphospho-sugar or sugar phosphate, to an acceptor molecule that is part of a growing polysaccharide chain (Campbell et al., 1997; 1998; Coutinho and Henrissat, 1999). The enzyme classification for GBSSI was initially EC 2.4.1.21 or EC 2.4.1.11, but the enzyme was reclassified to EC 2.4.1.242 when either UDP-glucose or ADP-glucose was shown to act as substrate (Shapter et al., 2009).

GBSSI in the *Poaceae* family are well conserved proteins encoded by the *Waxy* locus, which extends > 5 kb and carries 12 or 14 exons, of which the first exon is untranslated (Taira et al., 1995). Twelve exons are present in barley and wheat *GbssI* (Yan et al., 2000; Rohde et al., 1988), whereas maize, rice and potato *GbssI* have two extra exons (Klösgen et al., 1986; Visser et al., 1989; Wang et al., 1990). The two extra exons are present within the corresponding exons four and six of barley (Yan et al., 2000). The barley *Waxy* locus is located on chromosome 7H and encodes a 603 amino acid long polypeptide with a predicted 70 or 75 amino acids long transit peptide (Rohde et al., 1988; Domon et al., 2002a). Like other GBSSI produced in plants,

the mature barley GBSSI is about 60 kDa and accumulates within starch granules where it constitutes a major protein (Hylton et al., 1996).

Amylose-free starches in maize is caused by low *GbssI* expression due to various insertions of transposable elements within the gene (Wessler and Varagona, 1985; Klösgen et al., 1986; Liu et al., 2007), whereas mutations at the 5' splice site of Wx^b first intron results in waxy rice (Cai et al., 1998; Isshiki et al., 1998). In hexaploid wheat, variation in the number and composition of active *GbssI* alleles results in varying amylose concentrations in grain starch from normal (~25%) down to amylose-free (waxy) starch (Nakamuraet al., 1998). Some near waxy barley lines have a deletion within the *GbssI* promoter (Domon et al., 2002), which drastically reduces *GbssI* expression (Patron et al., 2002). A truncated GBSSI with no enzymatic activity is produced by waxy barley cultivar Yon M Kei due to a single-nucleotide substitution within the coding region (Patron et al., 2002). Other waxy barleys accumulate a full-length GBSSI within starch granules, but no amylose is produced (Ishikawa et al., 1995; Bhatty and Rossnagel, 1997). A mutation altering a critical residue at the active site has been suggested to underlie the amylose-free starch phenotype of barley genotype CDC Alamo (Patron et al., 2002).

A higher expression of *GbssI* leads to an increased (> 25%) amylose production in some Bangladesh rice cultivars (Jahan et al., 2002). Other high amylose starches are generally not due to mutations in *GbssI*, but rather other starch biosynthetic enzymes. Thus, various mutations affecting *Sbe2*, *Ss2* or *Ss3* expression or activity are known to increase amylose content in wheat (Yamamori et al., 2000, Regina et al., 2006), rice (Nishi et al., 2001) and maize (Boyer et al., 1976b, Gao et al., 1998) starches. For barley, an *amo1* mutation is responsible for the high amylose starch (~35%) produced by Glacier AC38 (Schondelmaier, 1992; Borén et al., 2008), but the gene underlying the mutation is not known. Barley *sex6* mutants produce starches with 65-70% amylose, but at the expense of low starch yields and shrunken kernels (Morell et al., 2003). Lack of SSIIa production with pleiotrophic effects on other starch biosynthetic enzymes is responsible for the *sex6* mutant phenotype.

To further improve our understanding of the effects of different *GbssI* mutations on amylose production in barley endosperm, a characterization of the *Waxy* locus in nine barley lines producing waxy, near waxy, normal, and increased amylose starches was undertaken in this study.

4.3 Materials and Methods

4.3.1 Plant material

Ten hulless barley (*Hordeum vulgare* L.) cultivars, previously characterized for carbohydrate content in grain (Asare et al. 2011) were used in the study. All genotypes accumulate a measurable amount of amylose in starch granules, except CDC Alamo and CDC Rattan, which produce waxy starch. Starches produced by genotypes CDC Candle, CDC Rattan and breeding line SB94912 contain low amount of amylose (4.5%, 4.5% and 1.6%, respectively), and are considered near waxy in this study. Genotype CDC McGwire has a normal starch phenotype with 25.8% amylose, whereas breeding lines SH99250, SH99073 and SB94893 produce starches with 38.5%, 40.8% and 38.0% amylose, respectively, and are classified as increased amylose lines.

Each genotype was grown separately in pots under controlled conditions at 22±1.0^{o}C and 320 µmol $m^{-2}s^{-1}$ photosynthetic photon flux density. Leaves were harvested at the 5-10 leaf stage, rinsed with distilled water and 70% (v/v) ethanol and quickly frozen in liquid nitrogen. The plant material was stored at −80°C and freeze-dried prior to DNA extraction.

4.3.2 Isolation of starch granules

Starch was purified from ten mature barley kernels with embryos removed using methods described by Zhao and Sharp, (1996) and Demeke at al., (1997) with minor modifications. The embryo-free seeds were steeped in 2 mL ice-cold water for 2 h and crushed in an eppendorf tube using a small pestle. Produced seed slurry (about 200 µL) was layered onto a 1.5 mL 80% (w/v) cesium chloride solution and centrifuged at 13,000 x g for 30 min. The pelleted starch granules were washed twice with 1 mL wash buffer (55 mM Tris-HCl pH 6.8, 2.3% (w/v) SDS, 10% (v/v) glycerol, 5% (v/v) β-mercaptoethanol), twice with 1.0 mL water, once with 1.0 mL acetone and air-dried overnight.

4.3.3 Extraction of Proteins Bound to Starch Granules

A 5-mg aliquot of dry starch granules were suspended in 150 mL sample buffer [62.5 mM Tris-HCl, pH 6.8, 2.3% (w/v) SDS, 10% (v/v) glycerol, 5% (v/v) β-mercaptoethanol, 0.0005% (w/v) bromophenol blue] and boiled for 5 min and immediately cooled on ice. Cooling time was at least 15 min to fully retrograde the amylose-free starches. The protein extract was

separated from gelatinized starch by centrifugation at 15,000 x g for 20 min, 4 ^{o}C and further cleared by an additional centrifugation at 15,000 x g, 20 min and 4 ^{o}C. A Bio-Rad Protein Assay kit (Bio-Rad Laboratories, Hercules, California, USA) based on the dye-binding assay of Bradford (1976) was used to determine protein concentration in extracts. The protein standard curve was produced using various concentrations of bovine serum albumin.

4.3.4 SDS-PAGE of starch granule-bound proteins

Samples of extracted proteins (5.0 µg) from starch granules were mixed with 20 µL sample buffer [62.5 mM Tris-HCl, pH 6.8, 2.3% (w/v) SDS, 10% (v/v) glycerol and 0.0005% (w/v) bromophenol blue] and loaded alongside a high range molecular weight standard (Bio-Rad Laboratories, Hercules , CA, USA) on an SDS-PAGE gel. The Protean II xi gel system (Bio-Rad Laboratories, Hercules , CA, USA) used consisted of 160 × 200 × 1.5 mm gels with 5% stacking gel (30:0.135 acrylamide:bis-acrylamide, 0.125 M Tris-HCl pH 6.8, 0.1% (w/v) SDS, 0.03% (w/v) ammonium persulphate, 0.07% (v/v) TEMED), 10% resolving gel [30:0.135 acrylamide:bis-acrylamide, 0.375 M Tris-HCl pH 8.8, 0.4 % (v/v) glycerol, 0.1 % (w/v) SDS, 0.03% (w/v) ammonium persulphate, 0.07% (v/v) TEMED] and running buffer containing 25 mM Tris-glycine pH 8.0, 192 mM glycine, 0.1% (w/v) SDS and 0.01% (w/v) β-mercaptoethanol.

Electrophoresis was performed at constant 20 mA for 16-18 h at room temperature. Silver staining of separated proteins was as described (Demeke et al., 1997). Pre-fixing of gel was done in 50% (v/v) methanol, 10% (v/v) glacial acetic acid for 30 min, and gel fixation was performed with 5.4% (v/v) methanol, 7.6% (v/v) glacial acetic acid for 30 min followed by three 10 min washes with water. The gel was incubated with 0.0005% (w/v) dithiotheitol for 30 min and stained with 0.1% (w/v) silver nitrate for 30 min. After washing the gel briefly in water for 10 sec, the polypeptide bands were developed in 3% (w/v) sodium carbonate, 1.8% (v/v) formaldehyde solution for 10-20 min before reaction was stopped by addition of citric acid to 2.3% (w/v) final concentration.

4.3.5 Immunoblotting

Polypeptides separated by SDS-PAGE were transferred by vertical electroblotting onto a Hybond nitrocellulose membrane (GE Healthcare Life Sciences, Piscataway, NJ, USA) using the Trans-Blot system of Bio-Rad (Bio-Rad Laboratories, Hercules, CA, USA). The transfer buffer consisted of 40 mM Tris-HCl pH 7.4; 20 mM NaAc; 2 mM EDTA; 50% (v/v) methanol; 0.05%

(w/v) SDS and transfer was done for ~13 h at constant 15 V, 4 ^{o}C. The membrane was incubated in blocking buffer [5% (w/v) non-fat milk; 1 × phosphate buffered saline, 0.1% (v/v) Tween 20] for 2 h and subsequently incubated with a 1:2,000 dilution of rabbit primary antibodies raised against wheat GBSSI (Demeke et al., 1999). Excess primary antibody was removed from membrane by four 15 min washes with blocking buffer. The membrane was subsequently incubated for 2 h with a 1:5,000 dilution of alkaline phosphate-conjugated goat anti-rabbit serum (Sigma-Aldrich Immunochemicals, St. Louis, MO, USA). Unbound secondary antibody was removed by three 10 min washes with blocking buffer and three 10 min washes with 50 mM Tris-HCl pH 7.5, 150 mM NaCl buffer. Immunoreactive polypeptides were visualized by chemical staining with 5-bromo-4-chloro-3-indolyl phosphate and 4-nitroblue tetrazolium using a BCIP-NBT kit (Bio-Rad Laboratories, Hercules, CA, USA). Semi-quantitative determination of signal intensity was done by densitometric scanning of immunoblot using the Gel-Doc Quantity One system (Bio-Rad Laboratories, Hercules, CA, USA).

4.3.6 DNA extraction

Genomic DNA was extracted from 200 mg freeze-dried leaf material using a 3% cetyl trimethylammonium bromide solution made up in 50 mM Tris-HCl pH 8.0, 1.4 M NaCl, 20 mM EDTA, 0.5% (w/v) polyvinylpyrrolidone and 0.2% (v/v) β-mercaptoethanol essentially as described by (Doyle and Doyle, 1987). The DNA quantity was determined by spectrophotometry using a DU800 UV spectrophotometer (Beckman Coulter, Brea, CA, USA).

4.3.7 PCR amplification of GBSSI gene fragments

Primers pairs specific to barley GBSSI locus were selected using the Vogelsanger Gold *Gbss1* nucleotide sequence (Genbank accession number X07932) and the Primer3 software (Rozen and Skaletsky 2000). The seven primer pairs used in the study are presented in Table 4.1 and their position on GBSSI gene is shown in Figure 4.1. The PCR reactions were performed in 50 µL volume containing 100 ng genomic DNA, 1.25 U Pfu Polymerase (Fermentas, Burlington, ON, Canada), 20 mM Tris-HCl (pH 8.8), 5 mM MgSO4, 10 mM $(NH4)_2SO4$, 10 mM KCl, 0.1 mg/ml BSA, 0.1% (v/v) Triton X-100, 0.5 mM dNTPs and 1.0 µM each of forward and reverse primers. The amplification conditions consisted of an initial denaturation step at 94 ^{o}C for 4.5 min followed by 32 cycles of 94 ^{o}C for 30 sec, 60 ^{o}C for 30-60 sec, 72 ^{o}C for 2.0 min, and a final incubation at 72 ^{o}C for 10 min. Five independent PCR reactions were performed per primer

Table 4.1. Barley *GbssI*-specific primers used in study**.**

Forward	Sequence	Position[1/]	Reverse	Sequence	Position[1/]	Amplicon (bp)
Hor_wx 1F	CAAAAAGCGAAGAGGAAGGA	261 - 280	Hor_*wx* 1R	AGAATCGAACCAACCGAGTG	1090 - 1071	830
Hor_wx 2F	CACTCGGTTGGTTCGATTCT	1065 - 1084	Hor_*wx* 2R	CACAGGGTGTGGCTACCTG	1796 - 1751	704
Hor_wx 3F	CGATCAGTAGCAGTCGTCTCTC	1536 - 1557	Hor_*wx* 3R	ACGCACCTTCTCCAGGAAC	2266 - 2248	731
Hor_wx 4F	TACAAGCGCGGAGTGGAC	2207 - 2224	Hor_*wx* 4R	ACGAGATGTTGTGGATGCAG	2911 - 2892	705
Hor_wx 5F	TTTTGCTAGGTGGCCTTCTG	2875 - 2894	Hor_*wx* 5R	TCCGATCACTCAATCATCCA	3593 - 3574	719
Hor_wx 6F	CTGATAGCTCCCCGTGAGG	3550 - 3568	Hor_*wx* 6R	CATTGAGCCATGCAGTCTTT	4244 - 4225	695
Hor_wx 7F	TTGGGATTTTCACAGCAATTT	4204 - 4224	Hor_*wx* 7R	CACCTCCCACCATCTTTGTT	5028 - 5008	825

[1/] Refers to *GbssI* sequence of Vogelsanger Gold (accession X07931).

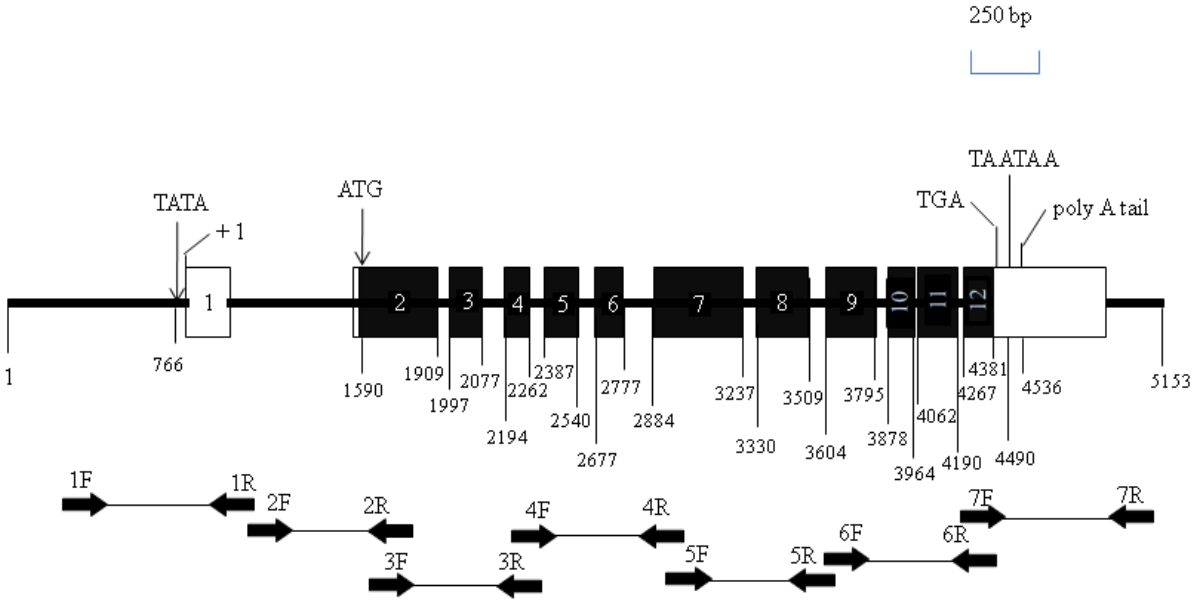


Figure 4.1 Structure of barley *Gbss1*
The illustration is based on *GbssI* sequence of Vogelsanger Gold (Rohde et al. 1988; accession X07931). Introns and non-transcribed regions are illustrated by a filled horizontal bar. Predicted exons are shown as boxes, where shadowed areas represent translated sequences. The position of predicted TATA-box, transcriptional start site (+1), start codon (ATG), stop codon (TGA), poly-A signal (TAATAA) and polyA tail are indicated. PCR amplicons generated to assemble *GbssI* contigs are outlined below.

pair and genotype. Amplified DNA fragments were separated by electrophoresis on 1% (w/v) agarose gels and isolated using a QIAquick gel extraction kit (Qiagen, Hilden, Germany). Quantification of isolated DNA was done using a DU800 UV spectrophotometer (Beckman Coulter, Brea, CA, USA).

4.3.8 DNA cloning and DNA sequence analysis

Purified DNA fragments were inserted into pJET1.2 vector using the CloneJET PCR Cloning kit (ThermoFisher Scientific, Waltham, MA, USA). Ligations were transformed into chemically competent *E. coli* DH5α cells as described (Sambrook et al., 2001) and plated on Luria-Bertani solid media containing 100 mg/mL ampicillin for growth overnight at 37 ^{o}C.

Transformed colonies were tested for desired insert by colony-PCR and one positive clone per independent PCR reaction was selected. The selected clones were propagated overnight in 100 µL 2YT media (1.6% Bacto-tryptone, 0.5% yeast extract, 0.5%, NaCl, 7.5% glycerol) placed in a 96-well microtiter plate at 37°C. The cultures were frozen at -80 °C and subsequently submitted to DNA Sequencing Service Centre, Plant Biotechnology Institute-National Research Council Canada, Saskatoon, SK for plasmid amplification and DNA sequence analysis.

The nucleotide sequences obtained from the seven DNA fragments of each *GbssI* allele were assembled into a single contig using the SeqBuilder module of the Lasergene software version 7.1 (DNASTAR, Madison, WI, USA). Alignments of contigs to *GbssI* sequences of barley genotypes Vogelsanger Gold (X07931), Morex (AF47373), Shikoku Hakada #84 (AB088761) and Muchimugi-D (AB087716) was done by Clustal W using the DNASTAR MegAlign module. To improve the alignments, small adjustments were done manually. Cleavage sites for transit peptides were predicted using the ChloroP 1.1 neural network (Emanuelsson et al. 1999; http://www.cbs.dtu.dk/services/). Searches for sequence similarities in the NCBI Genbank were done using the blastn tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Putative TATA-boxes were identified using plant *cis*-acting regulatory DNA elements (PLACE) database (http://www.dna.affrc.go.jp/PLACE).

4.4 Results and Discussion

4.4.1 Analysis of GBSSI accumulated in starch granules

The nine genotypes selected for the study show varying levels of amylose concentration in starch granules (Asare et al. 2011). To determine if the amount of GBSSI accumulated within starch granules correlated with amylose content, granule bound proteins were extracted from different starches and analyzed by SDS-PAGE. CDC McGwire producing starch with normal amylose concentration showed a prominent granule-bound protein of about 60 kDa (Figure 4.2A), which was confirmed to be GBSSI by immunoblot analysis using polyclonal antibodies raised against wheat GBSSI (Figure 4.2B). The wheat antibody reaction with barley GBSSI was expected as the primary structures of wheat and barley GBSSI are very similar (94% amino acid identity).

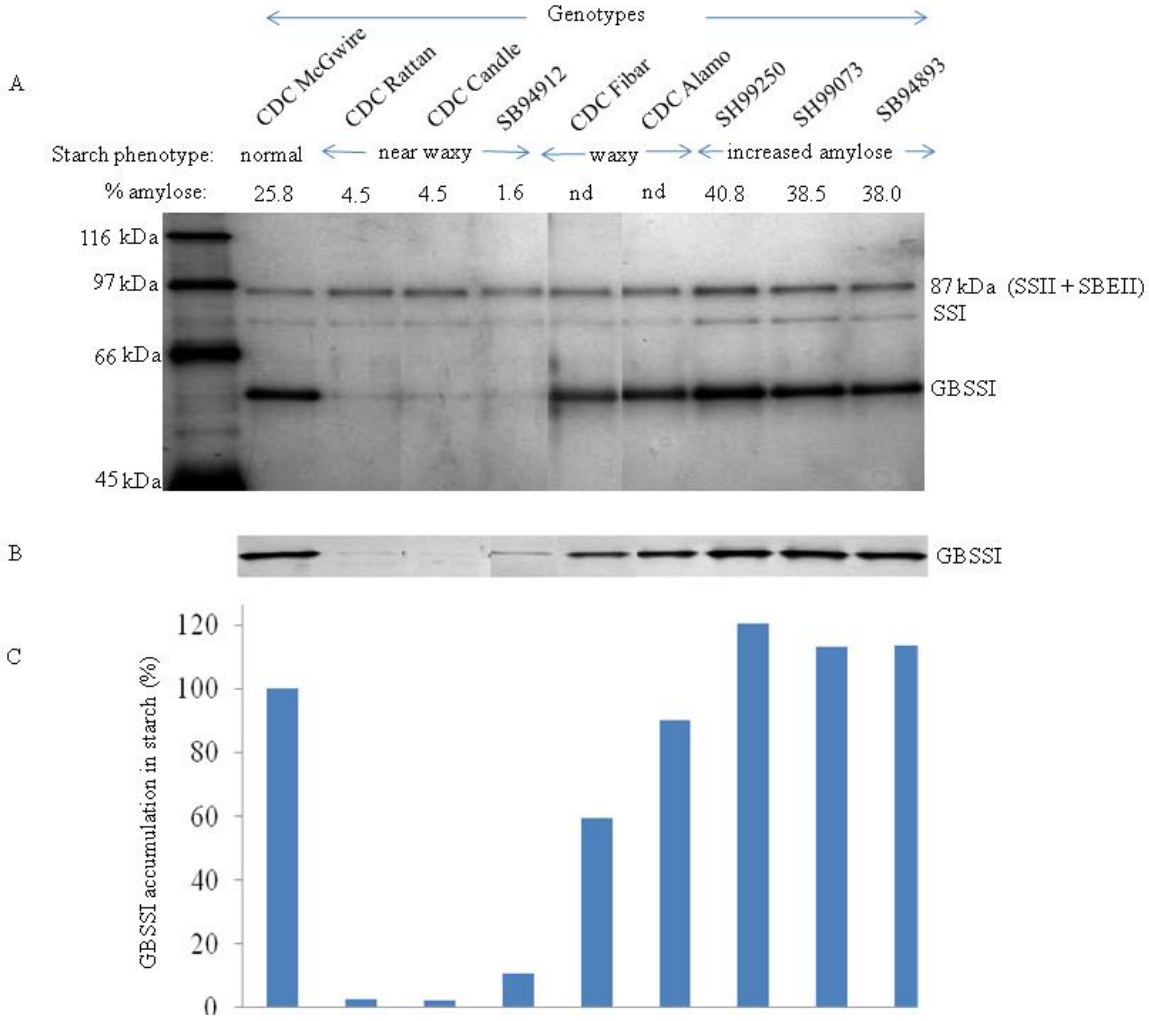


Figure 4.2 Analysis of GBSSI accumulation in endosperm starch granules.
(A) SDS-PAGE analysis of starch granule-bound proteins of barley genotypes with varying amylose concentrations. Molecular mass of protein standards is shown to the left and migration of known starch granule-proteins is shown to the right. (B) Immuno-reactive signal obtained with GBSSI antibodies. (C) Relative band intensity of immune-reactive bands with normal starch genotype CDC McGwire set to 100%.

Further analysis of SDS-PAGE and immunoblot revealed drastically reduced GBSSI levels in starch granules isolated from the three near waxy genotypes CDC Rattan, CDC Candle and SB94912 (Figure 4.2A-B). A densitometric scan of the immunoblot estimated GBSSI abundance in near waxy starches to be ≤ 10% of normal starch produced by CDC McGwire

(Figure 4.2C). Low *GbssI* expression or production of GBSSI isoforms that were labile or poorly incorporated into starch granules were possible reasons for the near waxy starch phenotypes. As compared to the three near waxy starches, waxy starches of CDC Fibar and CDC Alamo showed a much higher abundance of GBSSI (Figure 4.2A-B). For these two genotypes, the GBSS1 accumulation was approximately 60 and 90%, respectively, than normal (Figure 4.2C). Starches of the three genotypes with increased amylose concentration (SB99250, SB99073 and SB94893) showed a slightly higher abundance of GBSSI (10-15%) than normal (Figure 4.2A-C). However, the intensities of an 87-kDa protein band corresponding to migration of SBEII and SSII polypeptides (Li et al. 2003) and 71 kDa SSI (Borén et al., 2004) were also more intense than in the normal starch sample. One explanation for the higher abundance of certain granule-bound proteins in increased amylose starches could be due to more efficient extraction of granule-bound proteins from amylose-rich starches as compared to amylopectin-rich or normal starch. Alternatively, genes encoding starch biosynthetic enzymes including GBSSI were more efficiently expressed or encoded with higher affinity for glucan polymers in increased amylose genotypes.

4.4.2 Allele variants of *Gbss1*

The barley cv. Vogelsanger Gold *Gbss1* nucleotide sequence (Genbank accession # X07931; Rohde et al., 1988) was used to design seven sets of oligonucleotide primers (Table 4.1) for PCR amplification of *GbssI* DNA fragments. To facilitate assembly of *GbssI* contigs, the amplicons were designed to overlap each other by at least ≥ 20 bp (Figure 4.1). The *GbssI* sequences generated for all nine barley genotypes spanned from position 261 to 5,027 of Vogelsanger Gold *GbssI* sequence and included 1,329 bp upstream of translational start site (position 1,590-1,592) to 644 bp downstream of stop codon (4,381-4,383). The DNA sequences of the nine contigs and the corresponding sequences of published sequences of *GbssI* for Vogelsanger Gold, Morex, Shikoku Hakada and Mochimugi-D were aligned to identify relationships between the different alleles. When the 5'UTR segments (nucleotides 261-1589) were aligned, three main clades were found (Figure 4.3A).

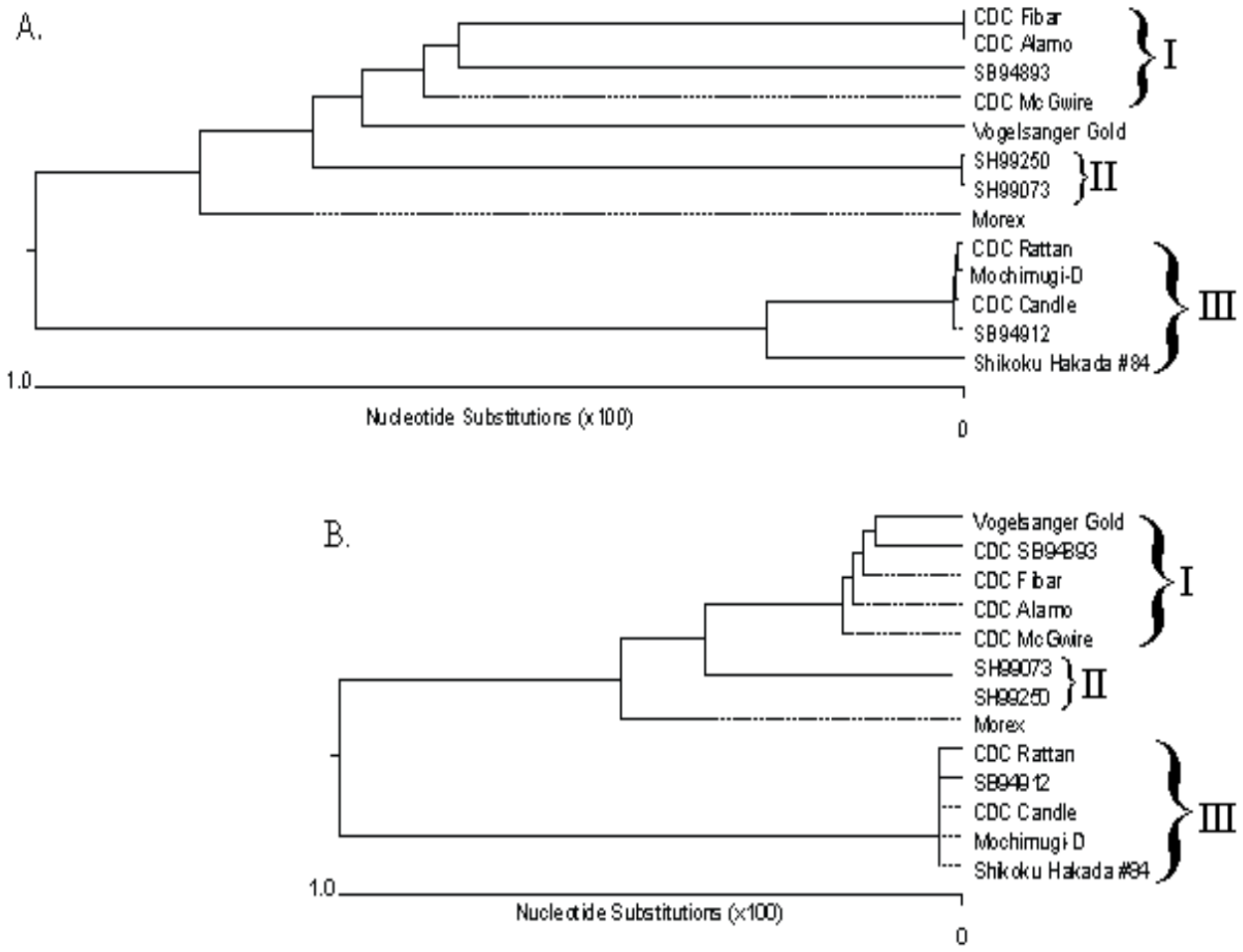


Figure 4.3 Phylogenetic tree showing relationships between different barley GBSSI genes. The trees were constructed using the Clustal W algorithm of DNAStar MegAlign module. Alignment of 5' UTR sequences is shown in (A) and gene segment from ATG start codon to stop codon in (B). The three clades proposed are indicated.

The first clade included sequences of waxy lines CDC Alamo and CDC Fibar, increased amylose genotypes SB9893 and normal starch genotype CDC McGwire. The second clade consisted of increase amylose genotypes SH99250 and SH99073, which were very closely related. Sequences of near waxy genotypes (CDC Rattan, CDC Candle, SB94912 and Mochimugi-D) grouped as clade three together with normal starch genotype Shikoku Hakada #84. The *GbssI* 5'UTR sequences of normal starch genotypes Vogelsanger Gold showed relationships to both clade I and II, whereas the Morex sequence related to both clade II and III.

When the sequence from start codon to stop codon (nucleotides 1,590-4,383) was analyzed, the sequence divergences were reduced between the alleles (Figure 4.3B). Essentially the same groupings were seen, with the exception of Vogelsanger Gold sequence, which became included into clade I. The sequence alignments of the two gene fragments showed that each genotype in the alignment carried a unique *GbssI* allele and suggested that subtle nucleotide differences for some of the genotypes could underlie the marked variation in GBSS1 accumulation and amylose concentration in grain starch.

4.4.3 *Gbss1* allele variants in genotypes with normal amylose concentration in starch

Alignment of the 4,840 bp contig of normal starch genotype CDC McGwire to sequences of normal *GbssI* alleles carried by Vogelsanger Gold, Morex, and Shikoku Hakada #84, respectively, was done to identify nucleotide changes that do not significantly alter amylose concentration in starch. This analysis revealed a total of 108 polymorphic sites along the *GbssI* sequence (Appendix 4.1). The CDC McGwire allele was most similar to that of Vogelsanger Gold (94% identity) and Morex (93.8%) identity, whereas Shikoku Hakada #84 was more divergent (91.8% identity). The Shikoku Hakada #84 sequence differences were mainly due to a 191-bp insertion 263 bp upstream of the ATG site and a 15-bp insertion within the transit peptide coding sequence. These modifications are often found in *GbssI* genes of Korean and Japanese germplasm with normal starch phenotypes (Domon et al., 2002a; 2002b).

Alignment of the deduced GBSSI amino-acids for the normal starch lines revealed very high sequence identities (Appendix 4.2). The 603 amino acid long pre-GBSSI produced by CDC McGwire was identical to that of Vogelsanger Gold. One amino acid preceding the transit peptide cleavage site differed between CDC McGwire and Morex pre-GBSSI sequences. Comparison to the Shikoku Hakada #84 genotype revealed several sequence differences, all located close to the amino-terminal end of the pre-protein (Appendix 4.2). The transit peptide cleavage sites predicted by the ChloroP 1.1 neuraal network (http://www.cbs.dtu.dk/services) were located between V_{69}-S_{70} residues of CDC McGwire and Vogelsanger Gold. For the Shikoku Hakada #84 and Morex pre-GBSSI, cleavage was postulated between V_{74}-R_{75} and V_{69}-R_{70}, respectively. Due to the 15-nucleotide long insertion within coding sequence of transit peptide, GBSSI produced by Shikoku Hakada #84 was five amino acid longer (74 amino acids) than the other three normal starch genotypes (69 amino acids). These predicted transit peptides were one residue shorter than determined for the amino-terminal end of purified barley GBSSI of

cultivar Satsuki-nijo (Taira et al 1995). It is possible that the pre-protein is cleaved after residue 69, but the first amino acid of generated protein is proteolytically removed as seen for several plant proteins (Huang et al., 1987, Kanno et al., 2007). Except for the initial amino acid predicted, the four normal starch genotypes produced identical 60 kDa mature GBSSI.

4.4.4 *Gbss1* allele differences associated with near waxy starch genotypes

The *GbssI* contigs generated for the near waxy lines (CDC Rattan, CDC Candle and SB94912) were aligned to the four normal *GbssI* allele sequences to find allele differences that were unique to the starch mutant genotypes (Appendix 4.1). Included in the alignment was also a *GbssI* sequence of near waxy barley genotype Muchimugi-D producing starch with 2.3% amylose (Yanagisawa et al. 2006). All unique nucleotide differences noted for the genotypes producing mutant starches were located to four exons (2, 6, 7 and 10) and three introns (1, 4 and 10) as presented in Table 4.2.

The near waxy genotypes in our study and Muchimugi-D were found to be near identical (99.8 %). They all shared a 403-bp deletion within the promoter region, a 191-bp insertion within intron 1 and a 15-bp insertion in sequence encoding the transit peptide (Appendix 4.2). When compared to Muchimugi-D GBSSI sequence, a few additional unique differences were noted for each of the CDC Rattan, CDC Candle and SB94912 sequences (Table 4.2). The 191-bp insertion and the additional five codons within the transit peptide were not expected to affect *GbssI* expression or activity as these insertion are also present in the *GbssI* of normal starch genotype Shikoku Hakada #84 (Patron et al., 2002). A more severe effect on *GbssI* expression was predicted from the 403-bp deletion, as it excluded the TATA box, transcriptional start site and untranslated exon 1 from the *GbssI* allele. Further on, the 403-bp deletion is associated with near waxy starch phenotypes in several six-row Korean and Japanese barley genotypes (Domon et al., 2002a) and is the only GBSSI sequence difference between near waxy Muchimugi-D and normal starch genotype Shikoku Hakada #84 (Domon et al., 2002a). Thus, it was concluded that the 403-bp deletion was the main reason for the near waxy phenotypes in CDC Rattan, CDC Candle and SB94912. The sequence alignment also suggested that the origin of the GBSSI alleles in Canadian near waxy genotypes is an Asian germplasm that is closely related to Muchimugi-D. A likely donor of the near waxy GBSSI allele is the Japanese line Murasaki mochi, which can be found in the pedigree of CDC Alamo (Patron et al. 2002).

Table 4.2. Unique allele differences in waxy, near waxy and increased amylose genotypes of barley[1/].

			Wild-types[2/]	CDC Rattan	CDC Candle	SB94912	Muchimugi-D	CDC Fibar	CDC Alamo	SH99250	SH99073	SB94893
Position[3/]	Region	Poly-morphism	normal starch	near waxy starch				waxy starch		increased amylose starch		
261	5'	SNP	C	C	C	C	C	G	G	C	C	C
313	5'	SNP	C	C	C	C	C	C	C	A	A	C
454	5'	SNP	C	C	C	C	C	C	C	C	C	T
559-560	5'	indel	C-A	C-A	C-A	C-A	C-A	C-A	C-A	CGA	CGA	C-A
584	5'	SNP	A	A	A	A	A	A	A	T	T	A
623-1026	5' + exon 1	variation	403, 408, 410, 419 nt	403 nt deletion	403 nt deletion	403 nt deletion	403 nt deletion	403 nt	403 nt	419 nt	419 nt	410 nt
1195-1198	intron 1	variation	ATGG / CTGG	CTGG	CTGG	CTGG	CTGG	CTGG	CTGG	CCGGG	CCGGG	CTGG
1206-1207	Intron 1	indel	T-C	T-C	T-C	T-C	T-C	T-C	T-C	TTC	TTC	T-C
1225-1226	intron 1	indel	G-A	G-A	G-A	G-A	G-A	G-A	G-A	GGA	GGA	G-A
1240	intron 1	SNP	G	G	G	G	G	G	G	C	C	G
1265	intron 1	SNP	A	A	A	A	A	C	C	A	A	A
1273-1274	intron 1	indel	G-C	G-C	G-C	G-C	G-C	GGC	GGC	G-C	G-C	G-C
1291-1292	intron 1	indel	G-C	G-C	G-C	G-C	G-C	G-C	G-C	G-C	G-C	CCG
1347-1348	intron 1	indel	TC	TC	TC	TC	TC	-C	-C	CT	CT	-C
1372	intron 1	indel	A-T	A-T	A-T	A-T	A-T	A-T	A-T	A-T	A-T	AAT
1389-1393	intron 1	variation	TTAAT / CCATT	CCATT	CCATT	CCATT	CCATT	TTAAT	TTAAT	TTAAT	TTAAT	TAATA
1399-1400	intron 1	variation	AG	AG	AG	AG	AG	AG	AG	AG	AG	GA
1429-1430	intron 1	indel	G-A	G-A	GGA	G-A	G-A	G-A	G-A	G-A	G-A	G-A
1694	exon 2	SNP	G/C	C	C	C	C	G	G	A	A	G
2267-2272	intron 4	variation	GACCGA	GACCGA	GACCGA	GACCGA	GACCGA	GACCGA	GACCGA	GACCGA	------	GACCGA
2274-2295	intron 4	variation	22 nt	22 nt	22 nt	22 nt	22 nt	22 nt	22 nt	22 unique nt	22 unique nt	22 nt
2297-2300	intron 4	variation	AGCT	AGCT	AGCT	AGCT	AGCT	AGCT	AGCT	AGCT	----	AGCT
2303-2307	intron 4	variation	CTTT	CTTT	CTTT	CTTT	CTTT	CTTT	CTTT	TTCCC	TTCTT	CTTT
2772	exon 6	SNP	G	G	G	G	G	G	G	G	G	A
2990	exon 7	SNP	A	A	A	A	A	A	T	A	A	A
3051	exon 7	SNP	G	G	G	T	G	G	G	G	G	G
3935	exon 10	SNP	G	G	G	G	G	T	G	G	G	G
3971	intron 10	SNP	T	C	T	T	T	T	T	T	T	T
4536-4546	3'	SSR	$a_{(11)}$; $a_{(12)}$; $a_{(15)}$	$a_{(14)}$	$a_{(14)}$	$a_{(15)}$	$a_{(15)}$	$a_{(12)}$	$a_{(12)}$	$a_{(12)}$	$A_{(11)}$	$a_{(12)}$

[1/] Polymorphism different from *GbssI* alleles of normal starch genotypes are highlighted. [2/] *GbssI* alleles of Vogelsanger Gold, Morex, Shikoku Hakada #84 and CDC McGwire. [3/] Based on *GbssI* sequence of Vogelsanger Gold (accession X07931).

Despite the lack of TATA-box signal and surrounding sequences, the near waxy genotypes were able to produce a small amount of GBSSI. This suggests that an alternate promoter was used for *GbssI* expression. A search for typical TATA-box signals in region upstream of ATG codon of *GbssI* carried by near waxy genotypes did not reveal any sequence matching typical promoter sequences present in the PLACE database (http://www.dna.affrc.go.jp/PLACE/index.html). However, a TATAAG motif matching the TATAΔ-PLM motif identified for a subset of plant promoters (Bernard et al. 2010) was noted for all *GbssI* alleles (nucleotides 1,372-1,377 in Vogelsanger Gold sequence). To determine if this potential alternative promoter is utilized in endosperm tissue, a full characterization of GBSSI transcripts produced in normal and starch mutant genotypes needs to be undertaken.

The nucleotide differences observed within intron 10 and at 3' end of CDC Rattan (Table 4.2) did not affect any sequence that appeared critical for *GbssI* expression. Nor could we find any critical region altered by two SNPs observed for CDC Candle within intron 1 and at 3'end, respectively (Table 4.2). Thus, these unique sequence differences noted for CDC Rattan and CDC Candle were not expected to cause additional effects on *GbssI* expression than the reduction caused by the 403 bp deletion in the promoter region. For SB94912, the 403-bp deletion and a unique SNP at position 3,051 were the only GBSSI sequence differences from the normal *GbssI* haplotype of Shikoku Hakada #84 (Table 4.2). The unique SNP identified created a *Sph*I restriction site within exon 7 and altered a glutamine residue to histidine in the SB94912 GBSSI sequence. The significantly lower amylose concentration in starch of SB94912 (1.6%) as compared to CDC Rattan (4.5%), CDC Candle (4.5%) and Muchimugi-D (2.3%), suggested that the Q312H change had a negative effect on GBSSI activity. A spontaneous mutation in a Muchimugi-D type *GbssI* allele or recombination with an allele encoding the H312 residue may have created the GBSSI haplotype of SB94912.

4.4.5 *Gbss1* allele differences associated with waxy genotypes

The sequence alignment of *GbssI* contigs and published sequences revealed five polymorphic sites between the *GbssI* alleles of waxy genotypes CDC Fibar and CDC Alamo and the normal *GbssI* haplotypes (Table 4.2). Four of the five sequence divergences were shared between CDC Fibar and CDC Alamo and were all located within a 107-bp region upstream of the TATAΔ-PLM motif (nucleotides 1,372-1,377) in intron 1. It is possible that one or several of

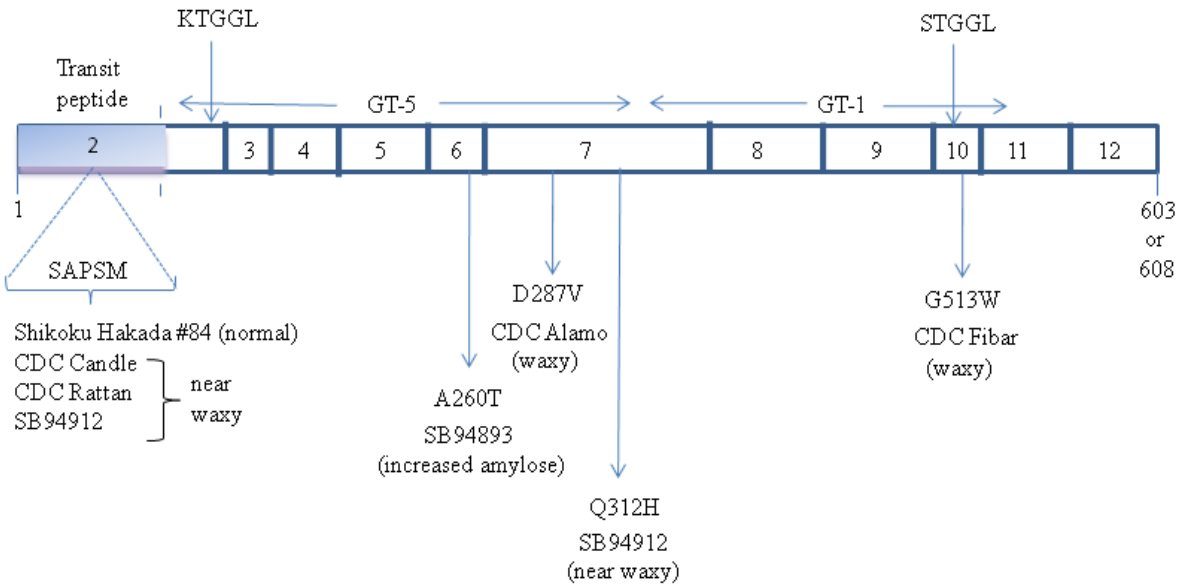


Figure 4.4 Amino acid variations observed for pre-GBSSI produced by different barley genotypes.
Vertical bar indicates pre-GBSSI with shadowed area representing transit peptide. Each block of the bar indicates the corresponding exon sequence. Location of glycosyltransferase domains 5 (GT-5) and 1 (GT-1) are indicated by horizontal arrows. Location of conserved KTGGL and STGGL motifs with possible role in catalysis and/or substrate binding are indicated (Edwards et al., 1999). The position of amino acid substitutions extending the transit peptide in germlines with normal and abnormal starches is indicated. Amino acids substitutions within mature GBSSI were all caused by nucleotide divergences from normal *GbssI* alleles.

these nucleotide changes affected *GbssI* transcription, which from a semi-quantitative analysis appears to be higher in CDC Alamo as compared to normal barley lines (Patron et al., 2002). Two unique sequence divergences for *GbssI* of CDC Alamo and CDC Fibar, respectively, were identified within exons seven and ten, respectively (Table 4.2).

An A2990T substitution in CDC Alamo introduced a *Drd*I restriction site and caused a D287V alteration in GBSSI amino acid sequence (Appendix 4.2; Figure 4.5). CDC Fibar carried a different SNP at position 3,935 producing a *Sex*A1 site and altering amino acid G513W in encoded GBSSI.

4.4.6 *Gbss1* allele differences associated with increased amylose genotypes

Many unique sequence differences for *GbssI* were found for the increased amylose genotypes (Table 4.2), which appeared to accumulate slightly increased amounts of granule-bound proteins within starch granules (Figure 4.2). Most of the *GbssI* sequence differences were common to genotypes SH99250 and SH99073 and positioned in 5' region and within introns 1 and 4. Genotype SB94893, which had its own set of unique sequence differences, also showed polymorphism within intron 1. The sequence differences within intron 1 are of interest as they could have a regulatory function for *GbssI* expression from the normal promoter and/or the alternate promoter. Whether temporal or spatial expression of *GbssI* is altered in increased amylose lines needs to be determined, before any conclusion can be drawn about the allelic differences observed. Nevertheless, one unique sequence difference from normal GBSSI was seen for GBSSI produced by genotype SB94893. A nucleotide variation at position 2,772 eliminated an *Eae*I site and altered A250T residue.

4.4.7 Structural features of barley GBSSI

The pre-GBSSI produced by a normal starch genotype CDC McGwire was 603 amino acids long, and the same number of amino acids was predicted for pre-GBSSI of increased amylose genotypes and the two waxy genotypes (CDC Alamo and CDC Fibar). A slightly longer pre-GBSSI (608 amino acids) was produced by the waxy genotypes CDC Candle, CDC Rattan and SB94912 due to a common 15-bp insertion within exon 1 (Figure 4.4). The extra five codons lie within sequence encoding the transit peptide, and also did not affect the transit peptide cleavage site. As the longer transit peptide is produced in barley lines with normal starch

composition (e.g. genotype Shikoku Hakada #84), the five extra amino acids within the transit peptide are not likely to affect targeting of GBSSI to plastids or abundance in starch granules.

Four different amino acid substitutions were identified for mature GBSSI (Figure 4.4). Three of the amino acid alterations were positioned within the glycosyltransferase 5 domain of GBSSI, whereas the fourth substitution was located to glycosyltransferase 1 domain (Figure 4.5). The single amino acid substitution A250T in GBSSI produced by increased amylose genotype SB94893 is positioned immediately in front of α-helix IH5 (Appendix 4.2). All GBSSI of barley, wheat, rice, Arabidopsis, *Chlamydomonas* and *Ostreacoccus* carry an alanine residue at this position, whereas isoforms of SSI, SSII and SSIII of the same species have alanine, serine, threonine, glycine or proline residues (Leterrier et al., 2008). Thus, the A250T could have a role in GBSSI enzyme specificity. The α-helix IH5 is followed by a loop denoted 380s, which is highly variable among starch synthases and speculated to be involved in the open-closed transitions upon binding of ADP-glucose (Leterrier et al., 2008). The conserved D287 residue within 380s loop is altered by the CDC Alamo D287V substitution, which may affect the catalytic properties of the enzyme (MacGregor, 2002). Therefore, the amylose-free starch produced by CDC Alamo is likely due to an inactive GBSSI as previously suggested (Patron et al., 2002). Close to the CDC Alamo D287V substitution, but outside of the 360s loop, lies the amino acid substitution Q312H observed for GBSSI of near waxy line SB94912. The polar uncharged amino acid change to a positively charged residue involves a conserved glutamine residue, supposedly important for binding to substrate or target. Despite the 403-bp deletion in the promoter region and the Q312H change in encoded GBSSI, the SB94912 genotype was able to produce a small amount of amylose. In contrast, the waxy genotypes CDC Alamo, which shows near normal GBSSI production did not produce any amylose. This suggests that the D287 residue is much more important for GBSSI activity than Q312.

The second domain of GBSSI was affected by a mutation carried by waxy genotype CDC Fibar. For this genotype, the amino acid change G513W is positioned in the C-terminal end that is unique to GBSSI enzymes (Figures 4.4, Leterrier et al., 2008; Edwards et al., 1999). This region of GBSSI carries a STGGL sequence that is similar to N-terminal KTGGL motif identified as binding site for ADPglucose in bacterial glycogen synthase (Furukawa et al., 1993). The substituted residue in CDC Fibar was positioned 10 residues downstream of STGGL motif

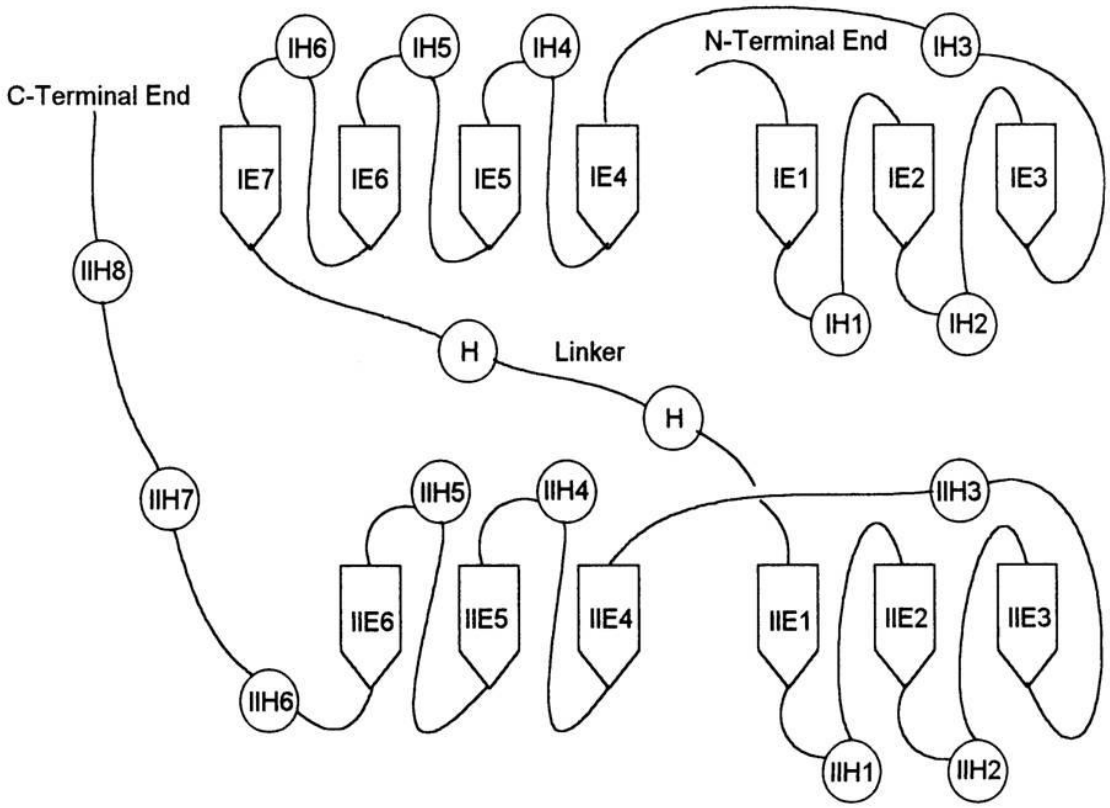


Figure 4.5 Schematic illustration of bilobed structure of glycosyltransferases.
The two glycosyltransferase domains are characterised by alternating α-helices (circles) and β-strands (arrows) which carry amino acids that make up the active sites (Campbell et al., 2000). The helices are numbered IH1 beginning from at the N-terminus and continue from the C-terminus as IIH1 to the number of helices. The β-strands like the helices are numbered similarly, however as IE1 and IIE1 respectively. A partially helical linker joining the two domains is shown as H - H. Three helices located at the C-terminal end bring the two ends of the polypeptide chain close to each other. (Figure is adapted from McGregor, 2002).

and it is the likely cause of GBSSI inactivity and production of amylose-free starch in the endosperm of CDC Fibar.

The mutation observed in CDC Alamo has been described earlier and exists in several barley genotypes (Patron et al., 2002). The other amino substitutions observed for GBSS1 of SB94912, CDC Fibar and SB94893 have not been described before.

4.4.8 Genetic markers for *Gbss1* mutant alleles

Genetic markers for genes of interest are important tools for breeding, as desired gene combination can be screened early in germplasm development. Thus, markers significantly reduce the time and cost for cultivar development and have become routine in breeding programs. To efficiently monitor *GbssI* alleles characterized in this study, four genetic markers for positive identification of mutant alleles were developed. The presence of the near waxy *GbssI* allele can be screened by PCR amplification using primer pair Hor_wx1F and Hor_wx1R (Table 4.1). In this assay, lines with the 403-bp promoter deletion (CDC Rattan, CDC Candle and SB94912) produced a 400-bp PCR product, whereas normal or increased amylose starch lines produced an 800-bp fragment (Figure 4.6A). The presence of the Q312H substitution, which potentially reduces amylose concentration in SB94912, can be identified by a cleaved amplified polymorphic sequence (CAPS) marker. PCR amplification with primer pair Hor_wx5F + Hor_wx5R, followed by restriction with *Sph*I is diagnostic for the SB94912 *GbssI* allele, which produced a 179-bp fragment in the assay (Figure 4.6B). The G513W amino acid substitution encoded by waxy germplasm CDC Fibar can be screened by using primer pair Hor_wx6F + Hor_wx6R and restricting the product with *Sex*AI to give a cleaved product if CDC Fibar *GbssI* mutation is present (Figure 4.6C). A CAPS marker for the D287V substitution encoded by *GbssI* of waxy germplasm CDC Alamo was obtained by PCR amplification with primer pair Hor_wx5F+Hor_wx5R and restriction with *Drd*I (Figure 4.6D). Production of a 606-bp DNA fragment was indicative of *GbssI* mutation carried by CDC Alamo. No positive diagnostic assay for mutation causing A250T substitution in GBSSI of SB94893 was tested. However, the normal *GbssI* alleles harbor a recognition site for endonuclease *Eae*I at the site of mutation in SB94893 *GbssI* allele. Thus, PCR amplification using primer pair Hor_wx4F and Hor_wx4R and restriction with *Eae*I would theoretically give a cleaved product for normal *GbssI* alleles, but not for SB94893 *GbssI* allele. In addition to genetic markers described above, the different alleles can be screened for using microsatellite markers flanking or located within *GbssI* transcribed region. Five useful microsatellelite markers for this purpose were identified from the sequence alignment (Table 4.3) and may be used to monitor *GbssI* alleles in marker assisted selection.

4.5 Conclusions

The detailed nucleotide sequence analysis of *Gbss1* from barley genotypes with varying grain starch amylose concentration revealed considerable nucleotide sequence heterogeneity.

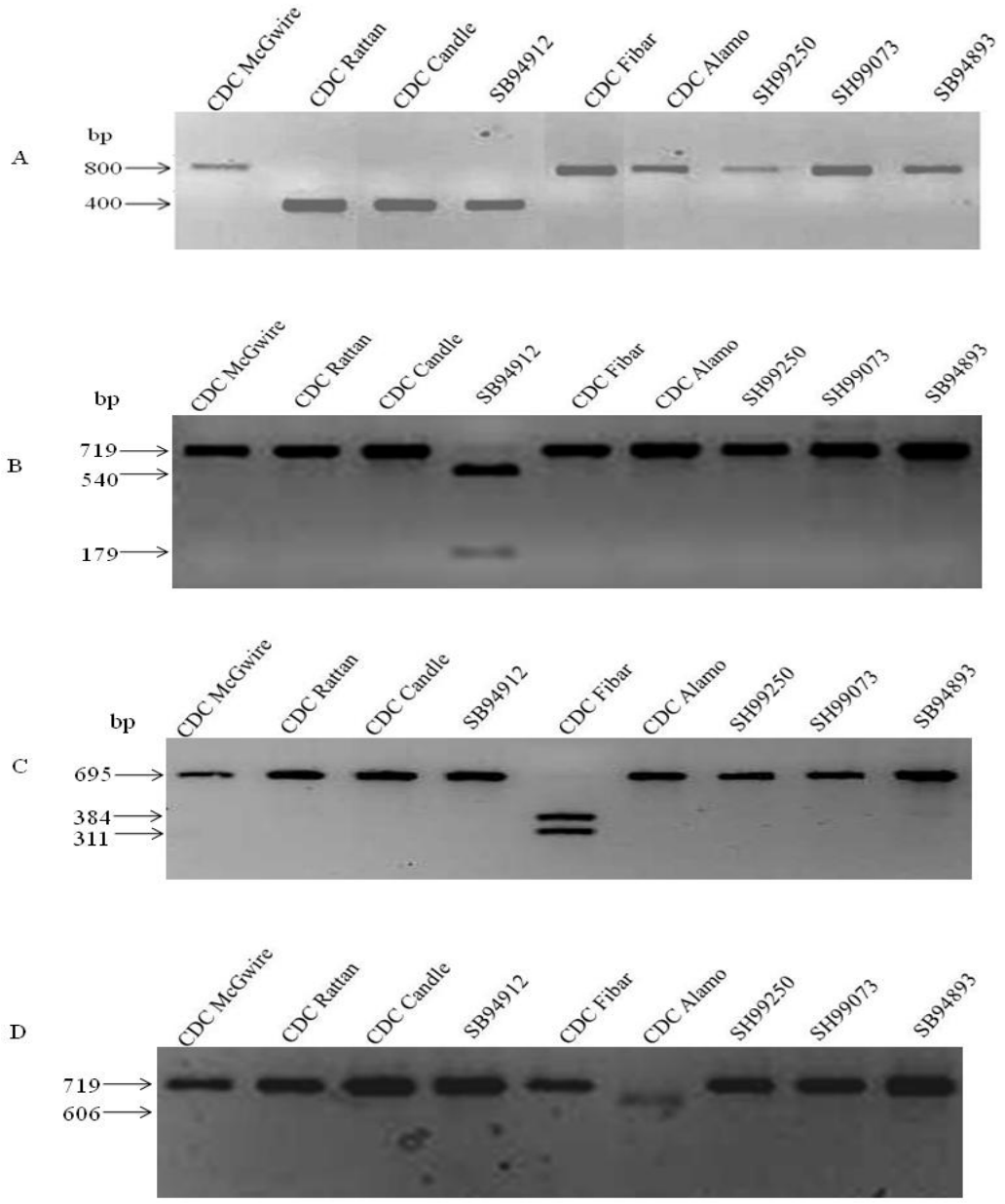


Figure 4.4 Genetic markers developed for *Gbss1*alleles.
PCR assay for promoter deletion in near waxy *GbssI* alleles carried by CDC Rattan, CDC Candle and SB94912 is shown in (A), Q312H substitution in GBSSI of SB94912 is shown in (B), G53H substitution in GBSSI of CDC Fibar in shown in (C) and D513V substitution of CDC Alamo is shown in (D).

Table 4.3. SSRs identified in study of *Gbss1*.

Postion[1/]:	367-378	704-716	2821-2836	4536-4546	4728-4734
Repeat:	(GA)n	(GGAGC)n	$(AT)_n$	$(A)_n$	$(T)_n$
Number of repeats:					
Vogelsanger Gold	6	1	9	11	7
Morex	6	3	13	12	7
Shikoku Hakada	7	3	9	15	8
CDC McGwire	8	4	9	12	7
CDC Rattan	7	-	9	16	7
CDC Candle	7	-	9	16	7
SB94912	7	-	9	17	7
CDC Fibar	8	4	9	12	7
CDC Alamo	8	4	9	12	7
SB99250	8	4	9	19	8
SB99073	8	4	9	19	8
SB94893	8	4	9	19	8
Muchimugi-D	7	-	9	15	8

[1/] Refers to position in Vogelsanger Gold sequence (accession X07931).

Three genotypes (CDC Rattan, CDC Candle and SB94912) showed severely reduced GBSSI production and amylose concentration due to a 403-bp deletion abolishing the main *GbssI* promoter, but not affecting a weaker alternative promoter. Similar promoter deletions have been reported for a few other near waxy barley genotypes (Patron et al., 2002; Domon et al., 2002a). The transit peptides encoded by the various genotypes were of different lengths, where a five amino acid longer transit peptide was proposed for pre-GBSSI of near waxy genotypes and the normal starch genotype Shikoku Hakada #84. However, the length of the transit peptide was not expected to have any effect on amylose production. Amino acid substitution D287V in Alamo and G513W in Fibar were identified as the likely causes of inactive GBSSI resulting in no amylose in starch granules. A Q312H substitution in GBSSI of near waxy line SB94912 may in addition to the promoter deletion reduce GBSSI activity and amylose production in endosperm to a very low level as compared to other near waxy genotypes.

Three genotypes (SB99250, SB99073 and SB94893) with increased amylose concentration seemed to accumulate a slightly increased amount of GBSSI and other starch biosynthetic enzymes in starch granules, but the reason behind the higher abundance could not be established from the study. The *GbssI* sequences of increased amylose genotypes displayed

many unique sequence differences within introns when compared to normal *GbssI* alleles. Whether these nucleotide alterations altered temporal or spatial expression of *GbssI* during endosperm development needs to be determined by additional studies of *GbssI* expression. It is also possible that mutation(s) in *GbssI* do not cause increased amylose phenotypes for any of the three genotypes studied. The increased GBSSI may merely be an effect of a mutation in another starch biosynthetic gene. As pleotropic effects are common for starch mutants (Cao et al., 1999; Edwards et al., 1999; Zhang et al., 2008), they may explain the simultaneous increase of GBSSI, SSI and 87 kDa polypeptides (SSII and / or SBEII) observed from SDS-PAGE (Figure 4.2). The altered starch granule profile observed for increased amylose starches in the study showed some resemblance to that of a rice high-amylose mutant, where both SSI and GBSSI production are increased due to SSIIa absence (Fujita et al., 2007). Increased levels of SSI, but also SSII and SBEIIb were seen in a proteome analysis of barley high-amylose mutant *amo1* (Borén et al., 2008). In contrast, reduced amounts of SSI, SBEIIa and SBEIIb in starch granules are observed in high-amylose *sex6* barley mutants, which lack SSIIa (Morell et al., 2003). Thus, variation in protein-protein interactions between different starch biosynthetic enzymes or phosphorylation status may underlie many of the pleotropic effects seen for starch mutants (Hennen-Bierwagen et al., 2009; Grimaud et al., 2008; Liu et al., 2009; Tetlow et al., 2008) and will be important to study in future experiments of increased amylose genotypes. To facilitate studies of *GbssI* expression, positive assays for four allele variants were developed in this study. These markers may also be useful for monitoring introgression of various *GbssI* alleles in barley improvement programs.

CHAPTER 5

CHARACTERIZATION OF *Sbe2b* GENES OF BARLEY GENOTYPES WITH VARYING AMYLOSE CONCENTRATION IN STARCH

5.1 Abstract

Nine barley genotypes producing starch with varying amylose concentration in starch granules were analyzed in the study. When compared to normal starch, the waxy and near waxy starches showed a low level of SBEIIb in granules, whereas SBEIIb levels were enhanced in increased amylose starches. A comparison of the *SbeIIb* nucleotide sequence determined for near waxy genotype SB94912, normal genotype CDC McGwire and increased amylose genotype SH99250 revealed a total of 21 polymorphic sites. All nucleotide differences were positioned within introns, and the processed SBEIIb transcripts produced by the three genotypes were predicted to be identical. The second intron of *SbeIIb*, which is known to regulate promoter activity, was identical between SH99250 and CDC McGwire, but SB94912 allele differed at 12 sites. Putative regulatory sites affected by the nucleotide differences within intron 1 were identified. Both starch mutants showed polymorphisms within *Sbe2b* introns 17, 18, 19, and 21 when compared to normal *Sbe2b* allele.

5.2 Intoduction

Starch produced in grain is composed of amylose and amylopectin, which are packaged in a 1:3 ratio into water-insoluble granules. The biosynthesis of starch is complex where various isoforms of starch synthases (SS), starch branching enzymes (SBE) and debranching enzymes (DBE) have a central role in the production of glucan polymers and definition of starch fine structure (Ball et al., 1996; Morell and Myers, 2005). Amylopectin biosynthesis involves several isoforms of SS, SBE and DBE, some of which are entirely soluble, whereas others are more or less associated with the starch granule (James et al., 2003; Nakamura et al., 2002). Each SS isoform extend their own set of available nonreducing ends on amylopectin, where production of the shortest chains is mainly due to SSI activity (Fujita et al., 2006). Chains of medium length are mainly produced by SSII (Umemoto et al., 2002; Morell et al., 2003; Yamamori et al., 2000), and the longest chains by GBSSI and SSIII activities (Ral et al., 2006; Fujita et al., 2007; Jeon et

al., 2010). Two types of SBE, SBEI and SBEII, are produced in endosperm tissue (Boyer and Preiss 1978). Both introduce α-(1→6) linkages in glucan chains, by cleaving α-(1→4) bonds and rejoining the cleaved glucans through its reducing end to C_6 hydroxyls to form α-(1→6) linkages (Borovsky et al., 1979). SBEI and SBEII differ in structure and catalytic properties, where longer branches are generally generated by SBEI activity and shorter branches by SBEII (Guan and Preiss, 1993; Takeda et al., 1993). The SBEII type is represented by two isoforms, SBEIIa and SBEIIb (Boyer and Preiss, 1981). In barley, *Sbe2a* is expressed in endosperm, embryo and vegetative tissues, whereas *Sbe2b* is exclusively active in the endosperm (Sun et al., 1998). Recent studies have demonstrated interactions between SSI, SSIIa, SSIII, SBEI, SBEIIa and SBEIIb *in vitro* supporting protein complex composition determines the final structure of amylopectin (Hannah and James, 2008; Hennen-Bierwagen et al., 2008; Tetlow et al., 2004, 2008).

Small glucans derived from amylopectin processing are believed to be the precursors for amylose biosynthesis (Denyer et al., 1996; van de Wal et al., 1998), which is catalyzed by granule-bound starch synthase I (GBSSI) alone (Shure et al., 1983). The low or amylose-free (waxy) starches produced by certain wheat, barley, maize and rice genotypes are generally due to mutations in *GbssI* (Cai et al., 1998; Isshiki et al., 1998; Klösgen et al., 1986; Nakamura et al., 1993; Taira et al., 1995). Highly active *GbssI* alleles have been shown to increase amylose concentration in certain Bangladesh rice lines (Jahan et al., 2002). Other increased amylose starches are not caused by increased amylose biosynthesis, but rather by increased synthesis of long glucan chains on amylopectin. These starches are due to mutations in genes encoding SBE or SS. For example, maize and rice *amylose extender* (*ae*) genotypes carry *Sbe2b* mutations and produces starches with greatly elevated amylose concentrations (Garwood et al., 1976; Mizuno et al., 1993). Lesions in maize *Sbe2a* causes increased amylose concentration in leaf starch, but does not affect endosperm starch composition in endosperm (Blauth et al., 2001). However, a simultaneous down-regulation of both *SbeIIa* and *SbeIIb* in wheat causes a 66 to 89 % amylose concentration in endosperm starch (Regina et al., 2010). Wheat and barley SSII mutants (Morell et al., 2003; Yamamori et al., 2000) and rice SSIII mutants (Fujita et al., 2007) also show an increase in apparent amylose concentration in endosperm starch.

In this study we characterized barley genotypes accumulating varying amounts of amylose, GBSSI and SBEIIb in starch granules. As mutations in *SbeIIb* may underlie the varying

SBEIIb production levels, the DNA sequence of SBEIIb gene was determined for a genotype with normal starch composition (CDC McGwire), a genotype with very low amylose concentration and SBEIIb content (SB94912) and a genotype with increased amylose and SBEIIb concentration (SB99250). Three different *SbeIIb* alleles were identified in the study.

5.3 Materials and Methods

5.3.1 Plant material

Nine barley genotypes accumulating various concentrations of amylose in starch granules of endosperm were used in the study. The characteristics of genotypes are summarized in Table 5.1. Production of plant material was as described in chapters 3 and 4.

5.3.2 Analysis of starch granule-bound proteins by SDS-PAGE and immunoblotting

Isolation of starch, extraction of granule-bound proteins followed by separation of granule-bound proteins by SDS-PAGE and silver staining of gels was as described in Chapter 4. Immunoblotting and scanning of blot were done as described in Chapter 4, with the exception of using wheat antibodies raised against wheat SBEIIb (Nair, 1997).

5.3.3 Assembly of *Sbe2b* sequences into contigs

A complete genomic sequence of barley *Sbe2b* has not been reported to-date. Therefore, design of *Sbe2b*-specific primers was based on a full-length barley *Sbe2b* mRNA (Genbank accession AF064561) combined with a partial SBE2b gene sequence (Genbank accession AF064563). To avoid selecting primers crossing a splice junction, *Sbe2b* mRNA sequence (Genbank accession AF064561) was aligned to *Aegilops tauschii* SBEIIb gene fragments (AY740398, AY740399 and AY740400) (Figure 5.1) using the Spidey software (http://www.genome.org/cgi). The sequence and location of primer pairs selected by the Primer3 design software (Rozen and Skaletsky 2000) are shown in Table 5.2 and Figure 5.2.

Genomic DNA and PCR amplifications were done according to protocols in Chapter 4. Five independent PCR products were generated for each primer pair, purified from agarose gel, quantified and cloned into pJET1.2 vector as described in Chapter 4.

Table 5.1. Characteristics of starch granules produced by genotypes in study.

Genotype	% Amylose[1]/	% β-glucan[1]/	Starch granule-bound proteins[2]/
CDC McGwire	25.8	5.1	Normal
CDC Rattan	4.5	7.4	Reduced GBSSI and SBEIIb
CDC Candle	4.5	6.9	Reduced GBSSI and SBEIIb
SB94912	1.6	8.2	Very low GBSSI and SBEIIb
CDC Fibar	nd	9.7	Reduced GBSSI and SBEIIb
CDC Alamo	nd	7.3	Reduced GBSSI and SBEIIb.
SH99250	38.5	7.9	Increased GBSSI, and SBEIIb
SH99073	40.8	8.5	Increased GBSSI, and SBEIIb
SB93893	38.0	7.9	Increased GBSSI, and SBEIIb

[1/] Asare et al., 2011. [2/] Chapter 3 and this study. Protein levels normalized to those of CDC McGwire.

5.3.4 DNA sequence analysis

DNA sequence analysis, assembly of contigs and sequence alignments were described in chapter 4. T-coffee multiple alignment program, was used to align the mRNA of *Aegilops tauschii* and mRNA of barley to determine the respective splice sites in barley (Notredame et al., 2000). The PLACE database (webpage) was used for searches of possible target sites for trans-acting factors.

5.4 Results and Discussion

5.4.1 Analysis of SBE2 accumulation in starch granules

Analysis of starch granule proteins by SDS-PAGE revealed a common profile for all genotypes studied; however, the band intensities were not uniform (Figure 5.3A). As reported in

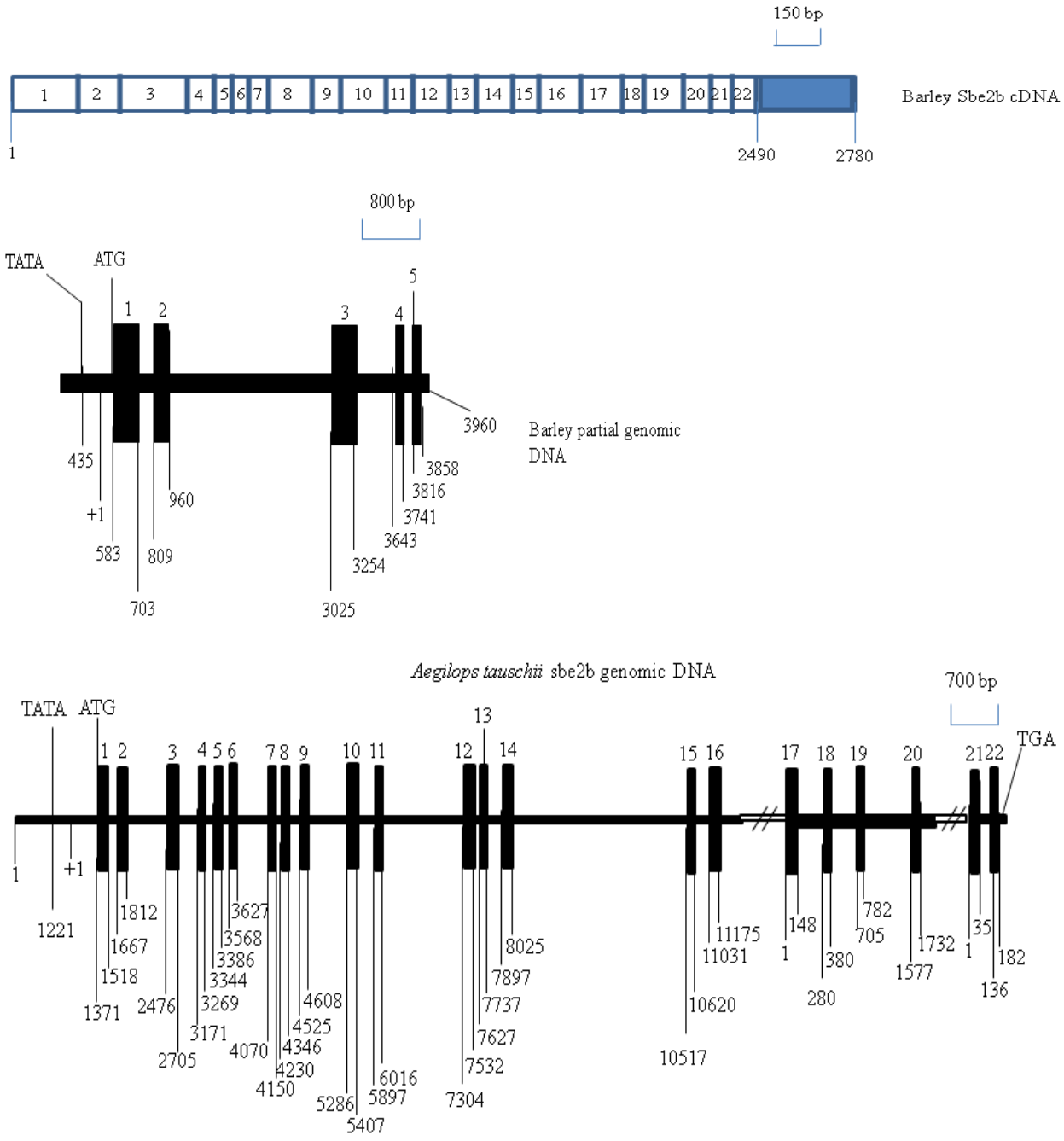


Figure 5.1 Schematic illustration of barley and wheat *Sbe2b* sequences.
(A) Barley *SbeIIb* cDNA of cultivar Bomi (Sun et al., 1998; accession AF064563) with exon numbers indicated. (B) Schematic organization of a barley partial *SbeIIb* sequence (Sun et al., 1998; accession AF064561). (C) Schematic organization of *Aegilops tauschii SbeIIb* gene fragments (Regina et al., 2005; accessions AY740398, AY740399 and AY740400). Gaps within introns 11 and 16 are shown by slashes.

Table 5.2. Gene specific primers for barley *Sbe2b.*

Primer	Nucleotide sequence (5'→3')	Nucleotides[1/]		Tm (°C)	GC (%)	Product (bp)
1F	GTTGATTGGTACCGACGCTT	Ctg1	1-20	60.5	50	494
1R	CGTCTCCTGCTTAAACCCAC	Ctg1	496-477	60	55	
2F	CTTGGTGGAAGATGTTCGGT	Ctg1	34-53	60	50	976
2R	AGTGAACCGTAAAAATGCGG	Ctg1	1011-992	58	45	
3F	CGTGGGTTTAAGCAGGAGAC	Ctg1	476-495	60	55	536
3R	AGTGAACCGTAAAAATGCGG	Ctg1	1011-992	59	45	
4F	CCGCATTTTTACGGTTCACT	Ctg1	992-1011	58	45	1657
4R	GACCACAACGCTCACTTGAA	Ctg1	2649-2630	60.5	50	
5F	GAAGATGAAACCCAAAAGTCG	Ctg1	2551-2571	58.5	43	993
5R	AAAGCAACACAGGGGAAATG	Ctg1	3545-3526	59.5	45	
6F	TATTTGTGCCACGGTTCTGA	Ctg1	2954-2973	59.5	45	974
6R	CAGACTGCAATTCGAAGCAG	Ctg1	3927-3908	61	50	
7F	ATAGGAGAATACGTTCAGACATTGA	Ctg1	3630-3648	57.5	36	509
7R	TGGATCCCAATTGTTGAAGTC	Ctg1	3962-3942	59.5	43	
8F	CTTACCGAGAATGGGCTCCT	Ctg1	3806-3825	60	50	980
8R	CGTGAGCCATGAGGAATTG	Ctg1	4500-4482	62.5	53	
9F	TTTTCTGCCAAACAATGCAG	Ctg1	4450-4469	58.5	40	1069
9R	TGGAAGCACCTCATCTCTGA	Ctg1	5894-5875	60	50	
10F	TCCATCTGGGACAAAGGATT	Ctg1	4601-4620	59.5	45	346
10R	CGCAATGATTTTGGTCGTTT	Ctg1	4943-4924	58.5	40	
11F	TGTATTCAAGCATCCTCAACCT	Ctg1	4902-4923	59	41	641
11R	TTGAACTGCATTGTATCCAAGTCT	Ctg1	5927-5904	57	38	
12F	TCAGAGATGAGGTGCTTCCA	Ctg1	5875-5894	62.5	50	526
12R	CATCAGGACAAGCAAACCAA	Ctg1	6553-6534	60	45	
13F	TTGGGTCCCCAGAAGATTTA	Ctg1	6486-6505	60	45	1208
13R	AGCCGCCATGAAAGTAATGT		nd	60	45	
14F	CTTGGACGGTTTGAATGGTT	Ctg2	1-20	60	45	279
14R	GAATCGGAAACCATCGAACT	Ctg2	271-256	60	45	
15F	CTTTCCAATGCAAGATGGTG	Ctg2	222-241	60	45	291
15R	ATCTACATCCGTGGCAAAGC	Ctg2	533-514	61.5	50	

Table 5.2. cont.

16F	CCATGAATATTTTGGCTTTGC	Ctg2	500-520	59.5	48	101
16R	CCAATAGTAACGGCTTCAGGA	Ctg2	601-581	59.5	48	
17F	TTGTTTACTTGATGCTGGTGAA	Ctg2	538-559	57	36	1504
17R	CAACGGCCATATGTAAGCGATA	Ctg2	1539-1518	60	46	
18F	GTTCAAGTTGGTGGGGTTG	Ctg2	1491-1509	62.5	53	637
18R	AACACACTTTTCCAACCACCTT	Ctg2	2102-2082	59	41	
19F	TGAAGGTTGGGAGATGGGTA	Ctg2	2037-2056	60	50	702
19R	ACCCTCTCCTCCTAAAGCCA		nd	63	55	
20F	TCATGATCAAGCACTTGTTGG		nd	59	43	577
20R	AGTGCTATTCCGCGATCAAT		nd	59	45	
21F	GAACGGACCTTCGACACCTA	Ctg3	1-20	61.5	55	118
21R	CCCGAACTCATTTCCCATAA	Ctg3	118-99	60	45	
22F	TCACAATGGCTTTAGGAGGAG	Ctg3	63-83	60	48	292
22R	GACGGCATTTGTCGTAACTG	Ctg3	342-323	62.5	50	
23F	GAGGCCCACAAGTACTTCCA	Ctg3	274-293	62.5	55	387
23R	TTCCTCAAGATGCTGCATTG	Ctg3	752-733	59	45	
24F	GCAGAATTTCTCAGGTATCATGG	Ctg3	690-712	58.5	44	652
24R	CAAGTCCCCTTTTTCAAACAC	Ctg3	1644-1624	59	43	
25F	CAATGCAGCATCTTGAGGAA	Ctg3	733-752	59	45	1363
25R	TGGATCCTACCAAATCCACC	Ctg3	3241-3222	60.5	50	
26F	ACTTGGTATTTGTGTTCAACTTCCA	Ctg3	1640-1664	57	37	776
26R	AGTGCTCTCCAGTGTGATGG	Ctg3	3258-3239	62.4	55	
27F	TCAGACGCTGGACTCTTTGG	Ctg3	3204-3223	62.5	55	229
27R	CTGTTAGTTCATTGGAGCATAGAC	Ctg3	3504-3481	58.5	40	
28F	ATGACAACAGGCCCCATTC	Ctg3	3380-3398	61.5	53	153
28R	TGCACCTGGTTGTATTGACC	Ctg3	3532-3513	60.5	50	
29F	CGCGCTGTTGTTGCTTAGTA	Ctg3	3477-3496	60.5	50	244
29R	TGGGAGAAGTATCGTTGCTG	Ctg3	3714-3695	60.5	50	

[1/] Refers to CDC McGwire *SbeIIb* contigs.

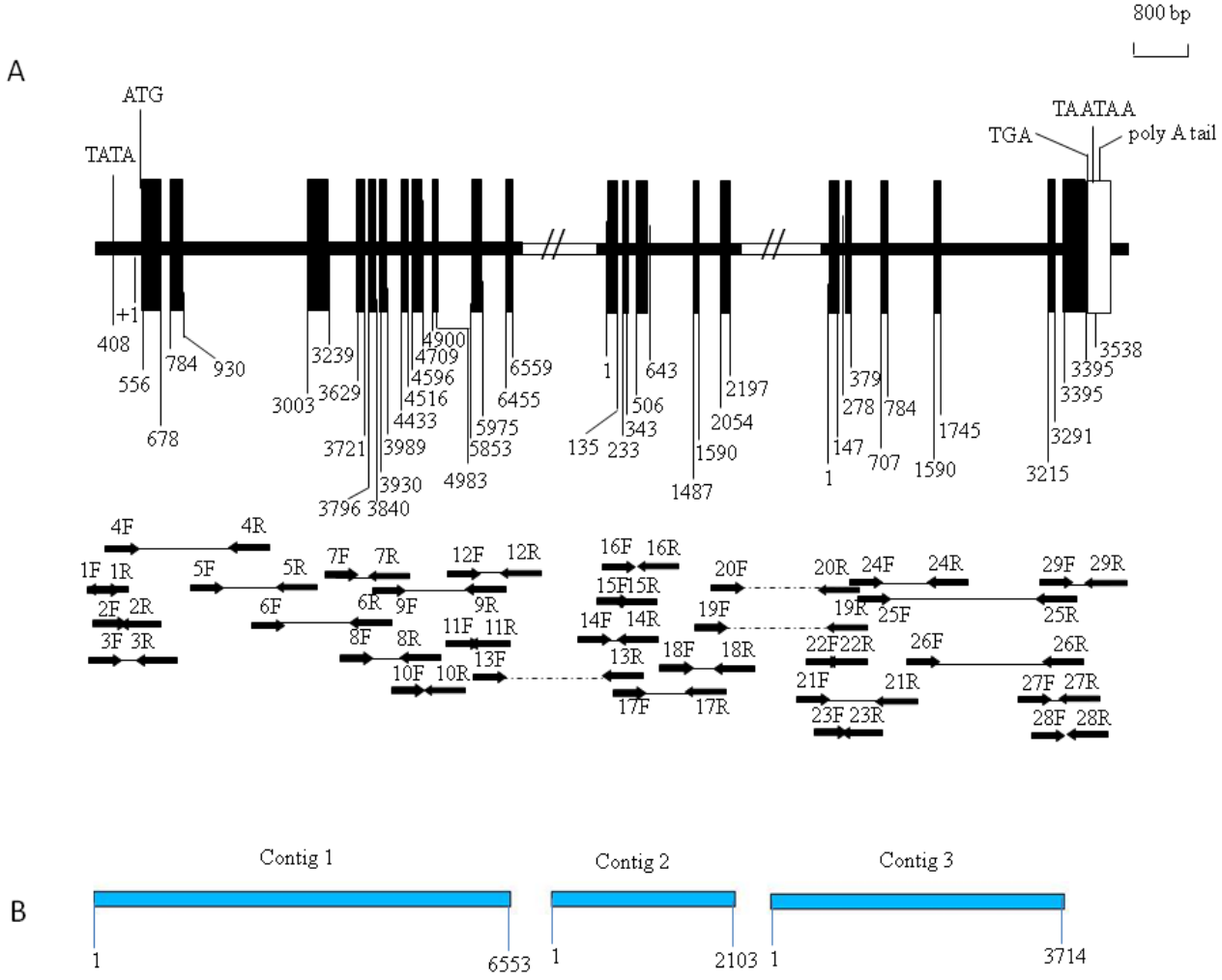


Figure 5.2 Schematic illustration of barley *Sbe2b*.
Vertical blocks represent exons, whereas the 5' non-transcribed region, introns and 3' region are shown by a horizontal line. (C) Position of TATA box, transcriptional start site (+1), translational start (ATG), translational stop (TGA) and polyA signal are shown. Below is shown PCR amplicons generated for SbeIIb to produce contigs 1, 2 and 2 as outlined.

chapter 4, the GBSSI abundance varied between the lines (Table 5.1) and in addition, the intensity of a 87 kDa granule-bound polypeptide varied. A weak 87 kDa polypeptide band was noted for starch of near waxy genotype SB94912 and a higher than normal abundance of 87 kDa band was seen in the three increased amylose starches (Figure 5.3A). A comparison to protein

profiles of starch granule bound proteins in barley suggested that the 87 kDa band contained SSII and SBEII polypeptides, which co-migrate on SDS-PAGE gels (Li et al., 2003).

An immunoblot analysis using antibodies raised against wheat SBEIIb was conducted, and confirmed immunoreaction with the 87 kDa protein band (Figure 5.3B). As revealed by SDS-PAGE, a weak immunosignal to 87 kDa protein band was noted for SB94912. Not so obvious from the SDS-PAGE, reduced amounts of SBEIIb polypeptides were also detected for the two near waxy genotypes CDC Rattan and CDC Candle and the two waxy genotypes CDC Alamo and CDC Fibar (Figure 5.3B). As compared to normal starch genotype CDC McGwire, the SBEIIb production in near waxy and waxy genotypes varied from 2 to 40% as estimated from a densitometric scan of the immunoblot (Figure 5.3C). In contrast, the increased amylose lines SH99250, SH99073 and SB94983 showed a 25-40% higher accumulation of SBEIIb than normal starch genotype CDC McGwire (Figure 5.3 B, C). With the exception of waxy starches, the SBEIIb abundance resembled that of GBSSI (Chapter 4, Table 5.1), which suggested that expression of SBEIIb and GBSSI were co-regulated. Alternatively, accumulation of the different granule-bound proteins depended on protein-protein interactions (Tetlow et al., 2004, 2008) or phosphorylation status (Satoh et al., 2008; Grimaud et al., 2008) of individual components of the starch biosynthetic complex. In an attempt to find the reason for the varying levels of SBEIIb in starch, a DNA sequence analysis of *SbeIIb* was undertaken. The genotypes selected for analysis were a normal starch genotype CDC McGwire, increased amylose genotype SH99250 with high GBSSI and SBEIIb production and near waxy genotype SB94912 producing very low amounts of GBSSI and SBEIIb.

5.4.2 Assembly of DNA contigs for barley *Sbe2b*

Barley *Sbe2b* contains 22 exons and the gene is estimated to be approximately 16.5 kb long (Sun et al., 1998; Fig 5.2A). To amplify *SbeIIb* gene fragments for DNA sequence analysis, twenty-nine oligonucleotide primer pairs were designed (Table 5.2) as outlined in Figure 5.2A. Overlapping *Sbe2b* amplicons were generated and assembled into larger contigs. Due to presence of large (> 4 kb) repetitive elements within introns 11 and 16, amplicons spanning these introns could not be obtained by PCR. An initial 4 kb sequence of intron 11 (data not shown) revealed the presence of repeat elements and further sequencing was discontinued.

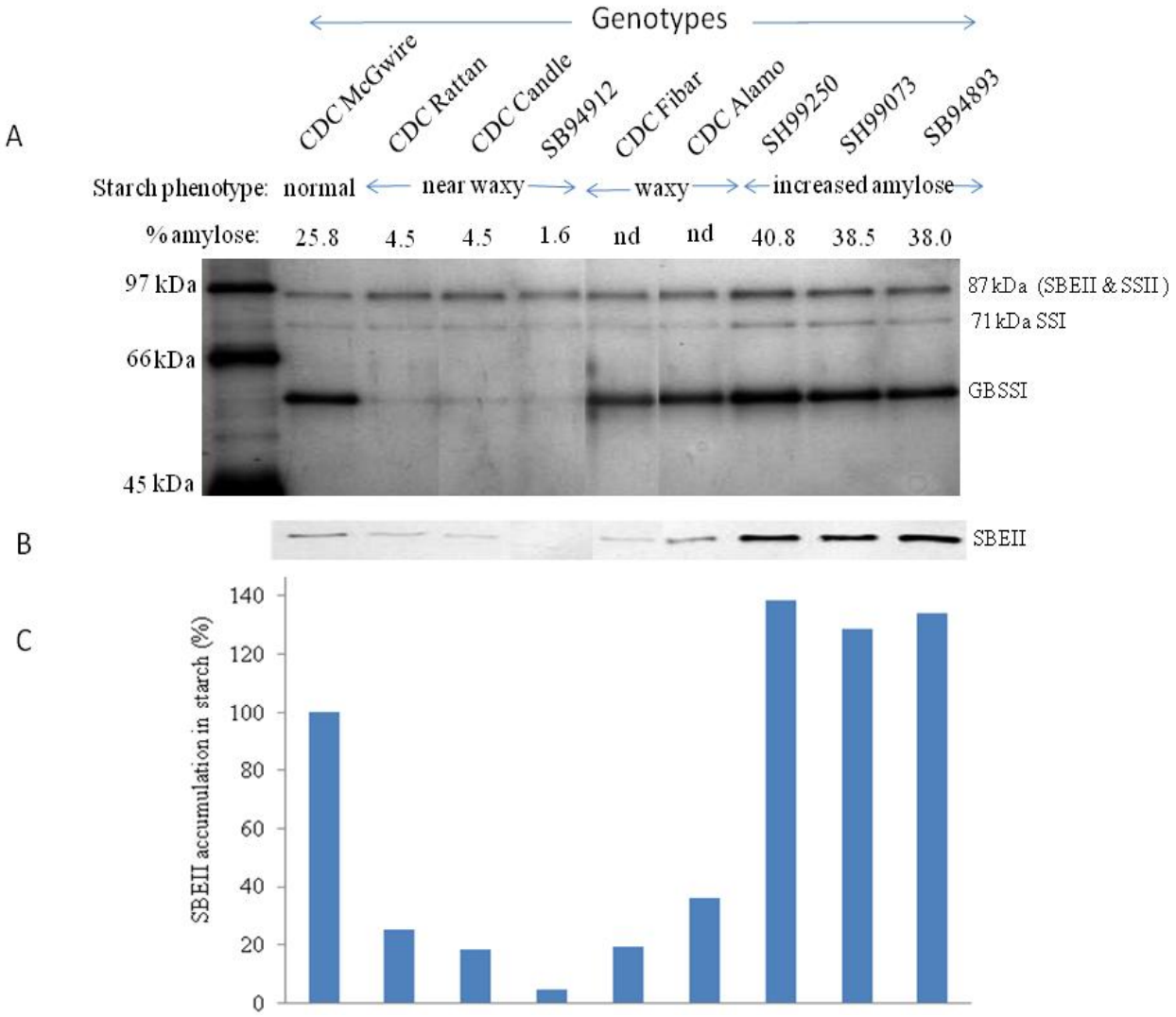


Figure 5.3 Analysis of SBEIIb accumulation in endosperm starch granules.
(A) SDS-PAGE analysis of starch granule-bound proteins of barley genotypes with varying amylose concentrations. Molecular mass of protein standards is shown to the left and migration of known starch granule-proteins is shown to the right. (B) Immuno-reactive signal obtained with SSIIb antibodies. (C) Relative band intensity of immune-reactive bands with normal starch genotype CDC McGwire set to 100%.

Thus, the sequences of the amplified *Sbe2b* genomic fragments were assembled into three contigs as illustrated in Figure 5.2B. The contigs spanned all *Sbe2b* exons, where contig 1 carried exons 1 through 11, contig 2 contained exons 12 through 16 and the last six exons were carried by contig 3.

5.4.3 Identification of polymorphic sites on *Sbe2b*

The DNA sequences of contigs generated for genotypes SH99250 and SB94912 were aligned to CDC McGwire contig 1 (6,553 nucleotides; Appendix 5.1) to identify nucleotide differences. For contig1, the SH99250 sequence was identical to that of CDC McGwire, whereas SB94912 sequence showed 12 polymorphic sites within intron 2 and one within intron 6 (Table 5.3). No difference was noted for contig 2 for the three *Sbe2b* alleles and this part of *Sbe2b* will not be discussed further. Alignment of the three contig 3 sequences revealed eight polymorphic sites (Table 5.3). Six sites with sequence differences were noted between CDC McGwire and SB94912, and six sites between CDC McGwire and SH99250.

There were no differences in exon sequences, nucleotides at exon/intron splice sites, the about 440-bp 5' non-transcribed region or the 264-bp 3' untranslated region. Therefore, the postulated *Sbe2b* alleles were presumed to produce identical mRNAs encoding a 91.3 kDa pre-SBEIIb protein. Thus, mutations affecting SBEIIb function were considered less likely to be the reason for varying levels of SBEIIb accumulated in starch granules.

5.4.4 Analysis of regulatory sequences within *Sbe2b* intron 2

The second intron of *SbeIIb* was found to be large (2,064 bp for McGwire and SH99250 and 2,069 bp for SB94912) as previously reported for *Sbe2b* of cultivar Bomi (2,064 bp; Sun et al., 1998). This intron is known to have a regulatory function for *Sbe2b* expression (Ahlandsberg et al., 2002) and carries a retrotransposon-like sequence that covers almost two-thirds of the intron (nucleotides 1150-2451; Appendix 5.1). This particular repetitive element is found in introns of several plant genes; for example the VRN-1 gene of wheat and barley, where it has a regulatory role (Fu et al., 2005).

Several of the nucleotide substitutions observed for genotype SB94912 alter the retrotransposon-like sequence; however the changes are limited to small indels and SNPs (Table 5.3). A previous study of *Sbe2b* expression have identified a B-box-like (*Bbl*) like sequence 'CTCCAAGACTCACCACA' within intron 2 (position 2,215-2,231). The motif is bound by nuclear factors present in leaves, but no interaction is seen with nuclear proteins of endosperm (Ahlandsberg et al., 1998). Thus, the *Bbl* element has been suggested to act as a repressor element in vegetative tissues where no S*be2b* expression can be detected (Sun et al., 1998;

Table 5.3. Unique polymorphic sites for germlines with altered starch composition

Contig	Position[1/]	Location	CDC McGwire Normal starch	SB94912 Near waxy starch	SH99250 Increased amylose
1	1316-1317	Intron 2	T—C	TTTC	T--C
1	1364	Intron 2	T	A[2/]	T
1	1724-1725	Intron 2	C—A	CCCA	C--A
1	1739-1740	Intron 2	C—G	CTTG	C--G
1	1773-1774	Intron 2	T-C	TTC	T-C
1	1789-1790	Intron 2	C-A	CCA	C-A
1	1817	Intron 2	G	A	G
1	1847	Intron 2	C	-	C
1	1936-1937	Intron 2	G-	GA	G-
1	1967-1968	Intron 2	G--	GTT	G--
1	2309-2312	Intron 2	TATC	CACA	TATC
1	2717-2721	Intron 2	CTGGA	-----	CTGGA
1	4215	Intron 6	G	T	G
3	224	Intron 17	G	T	G
3	232	Intron 17	T	T	G
3	235	Intron 17	A	A	G
3	238	Intron 17	G	T	G
3	250	Intron 17	G	T	T
3	664-676	Intron 18	TTTCTTGTTTTAC	TCTCTCGTTTTAC	AAACTTGAAACTT
3	1551-1560	Intron 19	GGCCATGTAA--	CCCCACCCCA--	GGGGATGGGGAT
3	3275-3284	Intron 21	TTTGCTCAGA	CTCGCTCTCG	TTTGTTTTGA

[1/] Position refers to nucleotides of contig 1 and 3 of CDC McGwire (Appendix 5.3). [2/] SNP variant also seen in *Sbe2b* allele of normal starch cultivar Bomi.

Table 5.4. Putative regulatory elements in *Sbe2b* intron 2 affected by allele differences.

Location[1/]	Motif	Sequence	Putative function	Strand	Presence	
					CDC McGwire SH99250	SB94912
1313-1318	ARR1AT	NGATT	ARR1 binding	+		yes
	POLLEN1LELAT52	AGAAA	Pollen specific expression	-		yes
1771-1775	DOFCOREZM	AAAG	Dof Zn-finger binding	-		yes
	POLLEN1LELAT52	AGAAA	Pollen specific expression	-		yes
1815-1818	SORLIP1AT	GCCAC	*phyA* (light)-induction	+	yes	
	CURECORECR	GTAC	copper-response element	+ / -		yes
1933-1938	WBBOXPCWRK1	TTTGACY	W-box; WRKY binding	+		yes
	QELEMENTZMZM13	AGGTCA	Pollen specific expression	-		yes
1965-1970	PALBOXAPC	CCGTCC	PAL cis-acting element	+	yes	
	MYBCOREATCYCB1	AACGG	MYB; cell cycle activation	-		yes
2309-2312	GATABOX	GATA	ASF-2 binding	-	yes	
2713-2723	INRNTPSADB	YTCANTYY	Initator element	+		yes
	GTGANTG10	GTGA	Pollen specific expression	-	yes	
	CACTFTPPCA1	YACT	*ppcA1* mesophyll expression	+	yes	
	ARR1AT	NGATT	ARR1 binding	+	yes	

[1/] Positions refer to nucleotide sequence of CDC McGwire contig 1.

Ahlandsberg et al., 2002). None of the nucleotide substitutions observed within intron 2 altered the *Bbl* element.However, neither of these elements or the *Bbl* element was affected by nucleotide substitutions observed in *SbeIIb* intron of SB94912.

A total of 318 motifs, many of which recognized by several factors, were obtained for CDC McGwire intron sequence. Those putative motifs affected by *Sbe2b* allele differences and are listed in Table 5.4. An inspection of these motifs suggests nucleotide variation for SB94912 led to acquisition of a W-box element within second intron. W-box elements are of interest as they are target sites for WRKY factors. The WRKY factor SUSIBA2 has a role for sugar responsiveness in endosperm, where it induces isoamylase expression by binding to sugar responsive element SURE (Sun et al., 2003). Like other WRKY, the factor can also interact with the unrelated W-box element (Sun et al., 2003). A functional SURE element has been postulated 206-bp upstream of *SbeIIb* TATA-box (Mutisya et al., 2006; Appendix 5.1); thus an additional SUSIBA2 binding site may be positioned within intron 2 of *Sbe2b* allele carried by SB94912. Whether this W-box sequence constitutes a functional repressor element within intron 2 needs to be determined.

5.4.5 Analysis of *Sbe2b* introns 17, 18, 19 and 21

The polymorphism identified within intron 17 were all SNPs. Larger sequence differences were noted within introns 18, 19 and 21 (Table 5.3), where the polymorphisms were positioned within a five to eight nucleotide distance from the splice sites. There is a possibility that the close proximity to the splice sites could have affected the efficiency of splicing for one or several of these introns.

5.5 Conclusions

A relatively low level of polymorphism was identified between the three *Sbe2b* alleles, for which nucleotide differences were limited to six introns. Nevertheless, the polymorphism can be utilized for development of allele-specific *Sbe2b* markers for future studies. The nucleotide analysis of *Sbe2b* contigs did not reveal any polymorphism that was a likely cause of low *Sbe2b* expression in waxy and near waxy lines or elevated expression in increased amylose lines. Although some nucleotide differences could possibly affect binding of regulatory factors or efficiency of splicing, the data presented here are insufficient to draw such conclusions. To

further the understanding of *Sbe2b* expression in the various starch mutants, a detailed transcriptional analysis needs to be undertaken.

It is also possible that polymorphism observed for *Sbe2b* alleles in the study do not affect *Sbe2b* mRNA levels. As SBEIIb forms complexes with other starch biosynthetic enzymes (Hennen-Bierwagen et al., 2008; Tetlow et al., 2008), the accumulation of SBEIIb within granules may be due to pleiotropic effects, where variations in other factors affect incorporation of SBEIIb into complexes and / or granules. In this context, it is interesting to note that phosphorylation of maize SBEIIb, GBSSI and starch phosphorylase is needed for these proteins to be granule-bound (Grimaud et al., 2008). Further on, SBEIIb absence in maize has been shown to alter the composition of complexes (Liu et al., 2009). A low starch phosphorylase activity could possibly explain the similar decrease of both GBSSI and SBEIIb in near waxy and waxy starches, whereas increased phosporylase activity would cause elevation of GBSSI and SBEIIb abundance in increased amylose starches. However, this hypothesis awaits further studies of phosphorylation status of granule-bound proteins and protein-protein interaction analyses.

CHAPTER 6

GENERAL DISCUSSIONS AND CONCLUSIONS

The current study focused on nine barley genotypes by (1) analyzing their major storage compounds (2) determining their starch physical properties and their effect on enzymatic starch hydrolysis (both in meal and extracted starch samples) (3) characterizing structural differences in *Gbss1* from nine genotypes and *Sbe2b* from three genotypes with varying carbohydrates concentrations. Nine barley genotypes in this study were classified into normal grain starch (CDC McGwire), near waxy or waxy grain starch (CDC Rattan, CDC Candle, SB94912, CDC Fibar and CDC Alamo) and increased amylose grain starch (SH99250, SH99072 and SB94893).

6.1 Influence of grain constituents and properties of starch on its enzymatic hydrolysis

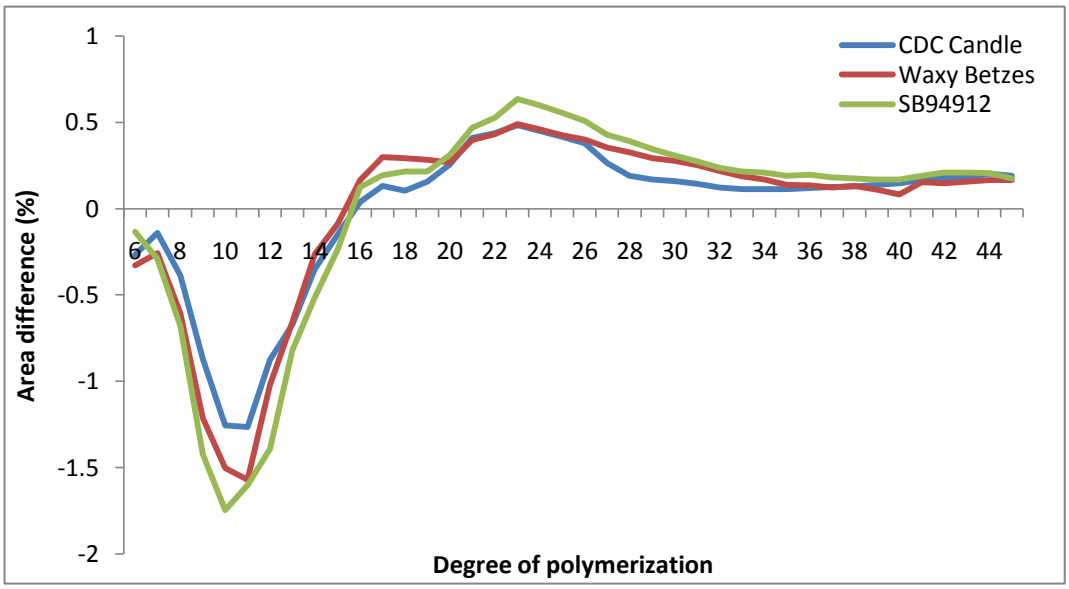
Barley is the fourth most important cereal after maize, rice and wheat in terms of production. (FAOSTAT 2009). Barley grain is a rich source of dietary fibers that have several health benefits. There is considerable interest to characterize barley grain constituents in diverse genotypes to increase their utilization for malt, feed and food (Griffey et al., 2010). To achieve this objective, detailed characterization of both physical and chemical properties of grain constituents and their influence in starch enzymatic hydrolysis was studied.

Grain starch amylose concentration strongly influences starch enzymatic hydrolysis, an important characteristic affecting barley grain utilization. Malt and beer production and animal feed, require low amylose starch that hydrolyzes easily. Whereas, human health benefits need starch with increased amylose and high beta-glucan concentration, that is less prone to enzymatic hydrolysis. The relative proportions of starch hydrolyzed during enzymatic digestion have been categorized into three classes: (1) rapidly digestible starch (RDS) (2) slowly digestible starch (SDS) and (3) resistant starch (RS). Rate of enzymatic starch hydrolysis has been correlated with starch physical properties including amylopectin chain length (Song and Jane 2000), starch granule sizes (Vasanthan and Bhatty 1996), amylose-lipid complexes and inherent enzyme inhibitors (Jane and Robyt 1984). Compared to normal wheat starch, waxy starch has higher crystallinity, transition temperatures and enthalpy (Demeke et al., 1999) and shows A-type X-ray diffraction pattern (Abdel Aal et al., 1997). Increased amylose starch shows B-type X-ray diffraction pattern and relatively lower crystallinity, transition temperatures and enthalpy (Song and Jane 2000) which suggest that the amylose fractions in increased amylose starch remain

amorphous. Starches with A-type X-ray diffraction patterns mostly cereal starches are easily hydrolyzed enzymatically compared with B-types. However, very limited information is available on the starch characteristics and other grain components influencing enzymatic hydrolysis of starch in barley kernel.

Change in starch composition (amylose and amylopectin concentration), also affects amylopectin structure. Mutations at the *wx* locus alter starch physical properties such as starch granules size and amylopectin chain length distribution (Konik-Rose at al., 2009). In wheat, waxy starch amylopectin has been shown to have long glucan chains (Yoo and Jane 2002). However, in barley, compared to normal starch genotype (CDC McGwire), three waxy starch genotypes (CDC Alamo, CDC Rattan and CDC Fibar) showed an increase in short glucan chains (dp 6-10), while in the other three waxy genotypes (CDC Candle, Waxy Betzes and SB94912) showed a decrease in short glucan chains (dp 6-14) but an increase in medium to long chain (dp 15-45) similar to that in wheat (Figure 6.1). The increased amylose genotypes had fewer short chains (dp 6-15) but increased medium to long chains (dp 15-40) compared to normal starch genotype (CDC McGwire) (Figure 6.1).

Amylopectin chain length distribution (CLD) described as F-I (dp < 12); F-II (dp 12-18) and F-III (dp 19-36) type chains and their packaging affect its physicochemical properties including enzymatic digestibility (Benmoussa et al., 2007). A positive correlation was observed between F-II chains and SDS concentration in pure starch samples ($r = 0.56$, $p < 0.01$). Amylose concentration positively correlated with RS in both meal and pure starch samples. Rate of starch hydrolysis in both meal and extracted starch samples followed the order waxy > normal > increase amylose. In the extracted starch samples, RDS was higher in waxy genotypes compared to normal starch genotype. The reason could be higher lipid concentration, in increased amylose (3.2 to 3.4%) compared to normal (3%) genotypes, which interferes with hydrolytic enzyme activity. Also waxy mutants with greater proportion of A-type granules have loosely packed granules which are readily attacked by hydrolytic enzymes contributing to faster rate of enzymatic starch hydrolysis in waxy genotypes compared to normal or increased amylose genotypes. The rate of starch hydrolysis was lower in meal samples compared to extracted starch. Reduced rate of starch enzymatic hydrolysis in meal could be due to the presence of enzyme inhibitors and interaction with other grain constituents such as lipids, β-glucan, dietary fiber and protein concentrations.



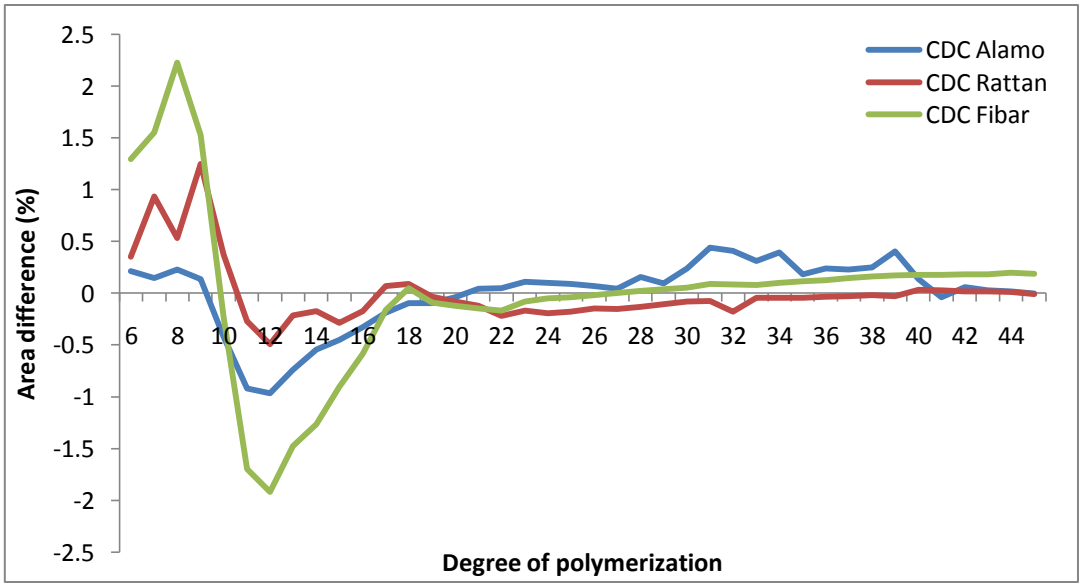
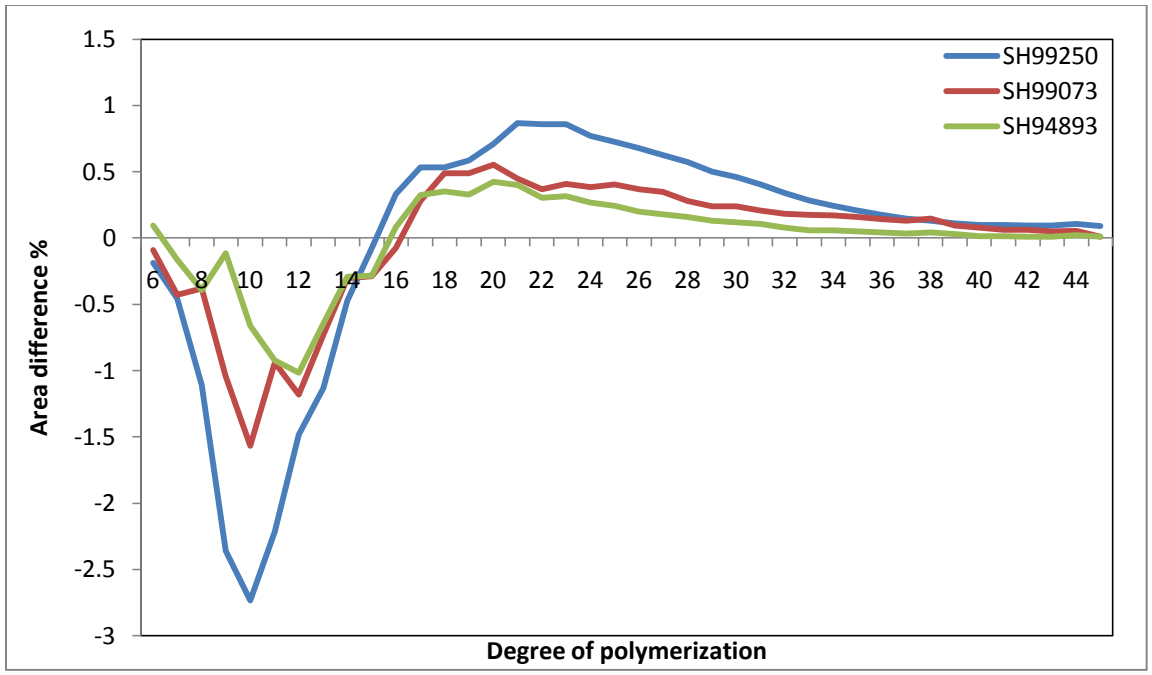


Figure 6.1 Comparison of amylopectin chain length profile of starches from barley genotypes with respect to CDC McGwire (normal starch genotype).
The percentage relative chain lengths were obtained by subtracting corresponding values from that of the normal genotype (CDC McGwire. The upper and lower graphs are for waxy genotypes.



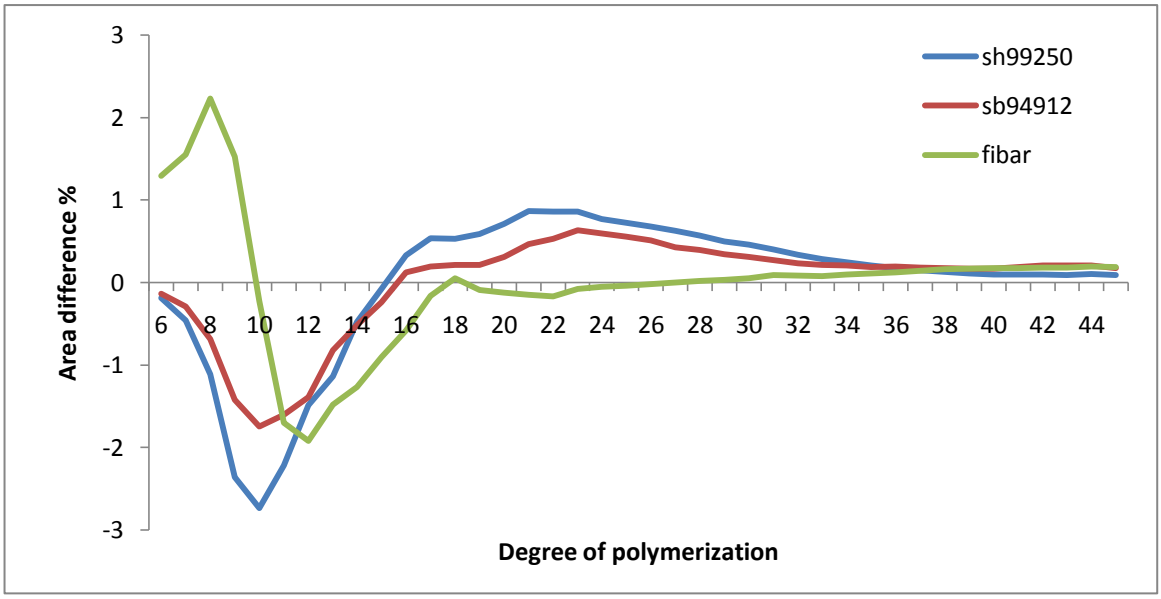


Figure 6.2 Comparison of amylopectin chain length profile of starches from barley genotypes with respect to CDC McGwire (normal starch genotype).
The percentage relative chain lengths were obtained by subtracting corresponding values from that of the normal genotype (CDC McGwire. The upper graph is for increased amylose whereas the lower graph compares an increased amylase to a waxy and near waxy genotypes.

6.2 Association of granule bound starch synthase I (GBSS I) and starch branching enzyme IIb (SBE IIb) with changes in starch structure

GBSSI is the only enzyme committed to amylose synthesis, therefore, it was present in very reduced amounts in three genotypes (CDC Rattan, CDC Candle and SB94912) with waxy starch (Table 4.1). However, two genotypes (CDC Fibar and CDC Alamo) had grain starch with non-detectable amylose, but GBSSI polypeptide was present (Table 4.1). Detailed nucleotide sequence analysis and predicted amino acid sequence revealed four single amino acid substitutions in the mature GBSS1 (Fig 4.4). Three of the amino acid alterations were detected in the glycosyltransferase 5 domain, while fourth substitution was located to the glycosyltransferase 1 domain. The D287V in CDC Alamo (Patron et al., 2002) and G513W in CDC Fibar (current study) were responsible for non-detectable amylose in endosperm starch. Aspartate and glycine are important for catalytic and substrate binding of GBSSI respectively (MacGregor 2002). In SB94912 Q312H made GBSS I inactive and potentially responsible for the reduced amylose concentration as Q312 is in the catalytic loop of the GBSSI enzyme (MacGregor 2002). The three genotypes (SH99250, SH99072 and SB94893) had increased amylose grain starch and also showed higher amount of GBSSI polypeptide as compared to normal grain starch genotype (CDC McGwire). However, only SB94983 showed A250T alteration, which is present immediately in front of α-helix IH5 (Appendix 4.2). This A to T transition could affect enzyme specificity, resulting in increased amylose phenotype. Alternatively, higher amount of GBSS I could be responsible for increased grain amylose in these three genotypes. The four single amino acid substitutions were used to develop DNA based markers to follow specific alleles in a marker assisted selection strategy.

Starch branching enzymes contribute to formation of α-(1→ 6) branching in a linear glucan chain to form branching glucan chains. Immunoblot analysis using SBEIIb specific antibodies revealed that five waxy grain starch genotypes showed reduced amount of SBEIIb whereas those with increased amount of starch amylose showed higher amount of SBEIIb compared to normal starch genotype (CDC McGwire) (Table 5.1). It is interesting to note that waxy barley genotype SB94912 had very low amounts of both GBSS I and SBE IIb polypeptide. Such a genotype can be considered as *amylose extender waxy* (*ae wx*) (Boyer et al., 1976b). Three genotypes CDC McGwire (normal grain starch), SH99250 (an increased amylose grain starch) and SB94912 (potential *ae wx*) were analyzed for *Sbe2b* nucleotide and predicted amino

acid sequences. The three genotypes showed no differences in exon sequences, nucleotides at exon / intron splice sites, about 440 bp non-transcribed region or the 264-bp 3' un-translated region. Therefore, the predicted *Sbe2b* alleles will produce identical mRNAs encoding a 91.3 kDa pre-SBE IIb protein. However, *Sbe2b* second intron was found to be large and showed variations between the three genotypes. The *Sbe2b* second intron has been shown to contain *Bbl* like sequences which through a negative repressor protein give endosperm specificity to SBEIIb (Ahlandsberg et al., 2002b). The *Bbl* sequence was present in all three genotypes studied. It is speculated that SB94912 endosperm may contain a repressor protein, which could cause low amounts of SBEIIb polypeptide. In summary, detailed *Gbss1* and *Sbe2b* analysis revealed the identification of a unique barley genotype (SB94912) which could be a potential *ae wx*.

Amylopectin glucan chain length distribution of normal starch genotype (CDC McGwire) showed that waxy starch genotype (CDC Fibar) had increased short chain (DP 6-10) glucan polymers, while the increased amylose genotype (SH99250) and a waxy starch genotype (SB94912) had reduced number of short chain (DP 6-14) glucan polymers (Figs 6.1 and 6.2). However, the two later genotypes had increased number of longer chains (DP 14-40), while a waxy grain starch genotype (CDC Fibar) had similar number as normal grain starch genotype (CDC McGwire). Rice *ae* and the *ae wx* starch also show a reduction in small chain but increase in long chain glucans (Kubo et al., 2010). The amylopectin structure results support the results obtained with starch granule proteins and the SBEIIb immunoblot analysis. The two independent lines of evidence suggest that SB94912 could be *ae wx* phenotype. This is the first report for *ae wx* barley, but more detailed studies need to be done to confirm this suggestion.

6.3 Future research directions

The study of nine barley genotypes with altered carbohydrate composition have revealed some interesting results regarding the two starch biosynthetic enzymes. Both the GBSSI and SBEIIb polypeptides were detected in higher quantities in the increased amylose genotypes. However, *Gbss1* promoter region comparison did not reveal any significant differences or identification of specific regulatory sequences that could cause increased GBSSI in the increased amylose genotypes. A detailed analysis of the promoter region and gene expression analysis may explain the increased amount of GBSSI in these genotypes. Absence of SBEIIb has shown subtle changes in amylopectin structure which needs to be further investigated. It will be of interest to completely characterize barley *Sbe2b* gene organization and expression and identify genotypes

with variant gene structure or expression. RNAi technology has shown that in barley, *Sbe2a* inhibition is needed to increase amylose concentration in both wheat and barley (Regina et al 2006; 2010). This is at variance from other cereal species such as maize and rice in which *Sbe2b* inactivity causes increased amylose concentrations. Results obtained in this research have shown that inactivity of SBEIIb and GBSSI can alter amylopectin structure. Altered amylopectin structure (increased dp19-36) affects starch enzymatic hydrolysis. This could be a new strategy to increase resistant starch concentration in barley grain and improve its health benefits. Availability of detailed gene structure and identification of functional motifs can be used to develop perfect gene based DNA markers which can be used in marker assisted selection to develop barley cultivars with desired carbohydrate concentration.

6.4 Conclusions and novel scientific contributions

The present study characterized nine barley cultivars with variable carbohydrate composition. Total starch concentration positively influences thousand grain weights. Protein concentration negatively influenced starch concentration and starch hydrolysis. Increased grain starch amylose concentration increased medium sized (5-15 μm) starch granules. F-III (DP 19-36) longer chain amylopectin which is associated with reduced enzymatic hydrolysis of starch.

Gene structure of *Gbss1* revealed a high degree of nucleotide sequence variability among the nine barley genotypes studied. In CDC Alamo a single amino acid substitution D287V (Patron et al., 2002) was confirmed and CDC Fibar identified a new amino acid substitution G513W as responsible for non-detectable amylose in the endosperm starch. In SB94912 another new amino acid substitution Q312H is responsible for reduced amylose concentration as Q312 is in the catalytic loop of the GBSSI enzyme (MacGregor 2002). In the increased amylose genotype SB94983 GBSSI, a single amino acid substitution A250T positioned immediately in front of α-helix IH5 is reported for the first time. This single amino acid substitution could alter GBSS1 enzyme substrate specificity to produce an increased amylose phenotype. Several new microsatellite and SNP markers have been identified to discriminate *gbss1* alleles in barley.

Three nucleotide sequence contigs spanning 14.5 kb of barley *Sbe2b* are reported for the first time. The presence of *Bbl* sequence in intron 2 is confirmed in all the three genotypes studied. Although no significant nucleotide sequence variability was observed in the *Sbe2b* sequence, however compared to CDC McGwire and SH99250, SB94912 intron 2 showed numerous alterations in nucleotide sequences.

Barley genotype SB94912, due to the absence of both GBSSI and SBEIIb polypeptides, and distribution of amylopectin glucan chain length suggest it as an amylose extender waxy (*ae wx*) genotype which is being described for the first time in barley.

Increasing barley utilization for feed, food and malting depend mainly on consumers demand for specific genotypes due to variations in storage composition. The normal genotype CDC McGwire has higher starch concentration (~70%) and lower dietary fiber (~5%) which makes it a suitable feed grain for monogastric animals such as poultry and pigs. The relative lower dietary fiber concentration reduces addition of fiber digesting enzymes such as beta-glucanase to monogastric animal feed. CDC McGwire can also serve as a suitable parent to increase the starch cenentration of an otherwise novel genotype with altered starch concentration. The waxy genotypes are ideal for industrial applications which demand 100% amylopectin starch. They are also best suited for food uses in famine prone regions that demand rapid release of glucose to meet the energy demands in humans. Contrarily, the waxy genotypes may not make high energy feed due to the high dietary fiber (β-glucan) and low starch concentration. CDC McGwire can be used as a parent in a cross to reduce the β-glucan concentration as well as increase the starch concentration to improve its potential as animal feed and food. The other two near waxy genotypes (CDC Candle and CDC Rattan) can also be utilized in the same way as the waxy genotypes.

Recent increase in health awareness has prompted healthy eating in which grain crops such as barley has been accepted for control of cardiovascular and colo-rectal diseases and weight control. The increased amylose genotypes (~38% amylose) also have high dietary fiber concentration. It is therefore expected that genotypes such as SH99250, SH99073 and SB94893 would be promoted for food uses to control the above mentioned metabolic diseases. Increased amylose concentration in cereals including barley is known to be due to up regulation of *Gbss1* / or down regulation of either *Ss2a* or *Sbe2b*. The three increased amylose genotypes also showed relatively higher amount of GBSSI polypeptide and whether that is due to higer *Gbss1*expression is yet to be tested. Down regulation of *Sbe2b* as observed in genotype SB94912 may also be used to increase starch amylose concentration. However this genotype has reduced GBSSI protein which can be improved by using any of the increased amylose genotypes as a final parent. Barley genotype CDC Hilose (http://www.inspection.gc.ca) with >40% amylose developed with SH99250 (> 38%) and CDC McGwire (25% amylose) as parents.

REFERENCES

AACC International. "Approved Methods of the AACC". Methods 76-11; 46-30; 32-07; 30-25. St Paul. MN

AACC (2001). The definition of dietary fiber. Cereal Foods World. Vol 46 (3) pp112-126

Aastrup, S and Jørgensen, K. G (1988). Application of the calcofluor flow injection analysis method for determination of β-glucan in barley, malt, wort, and beer. Journal of American. Society of Brewing Chemist. 46: 76-81

Abd Allah, M. A., Foda, V. H., Mahmoud, R. M and Abou Arab, A. A (2006). X-Ray diffraction of starches isolated from yellow corn, sorghum, sordan and pearl millet. Starch - Stärke 39(2): 40-42.

Abdel Aal, E. S. M., Hucl, P. J and Sosulski, F. W. (1997). Structural and compositional characteristics of canaryseed (*Phalaris canariensis* L.). Journal of Agriculture and Food Chemistry. 45: 3049–3055.

Åberg, E (1938) *Hordeum agriocrithon* nova sp., a wild six-rowed barley. Ann Agric Coll Sweden 6:159–212.

Abel G. J. W., Springer, F. and Kossman, J. (1996). Cloning and functional analysis of a cDNA encoding a novel 139 kDa starch synthase from potato (Solanum tuberosum L.). Plant Journal. 10, 981-991

Adams, C. L., Hambidge, M., Raboy, V., Dorsch, J. A., Sian, L., Westcott, J. L and Krebs, N. F (2002). Zinc absorption from a low–phytic acid maize. American Journal of Clinical Nutrition 76:556–9

Ahlandsberg, S., Sun, C and Jansson, C (2002). An intronic element directs endosperm-specific expression of the *sbeIIb* gene during barley seed development. Plant Cell Report 20:864–8.

Ainsworth, C., Clark, J and Balsdon, J (1993). Expression, organisation and structure of the genes encoding the *waxy* protein (granule-bound starch synthase) in wheat. Plant Molecular Biology 22: 67–82

Albertsson, A. C and Karlsson, S. (1995). Degradable polymers for the future. Acta Polymerica, 46, 114–123

Åman, P and Newman, C. W (1986). Chemical composition of some different types of barley grown in Montana, USA. Journal of Cereal Science 4: 133-141.

Åman, P., Hesselman, K and Tilly, A. C (1985). Variation in the chemical composition of Swedish barleys. Journal of Cereal Science. 3:73-77.

Ames, N., Rhymer, C., Rossnagel, B., Therrien, M., Ryland, D., Dua, S and Ross, K. (2006). Utilization of diverse hulless barley properties to maximize food product quality. Cereal Foods World 51, 23–38.

Andersen, K. E., Bjergegaard, C., Møller, P., Sørensen, J. C. and Sørensen, H (2005). Compositional variations for r-Galactosides in different species of leguminosae, brassicaceae, and barley: A chemotaxonomic study based on chemometrics and high-performance capillary electrophoresis. Journal of Agriculture and Food Chemistry. 53:5809-5817.

Andersson, A. A. M., Andersson, R and Åman, P (2001). Starch and by-products from a laboratory-scale barley starch isolation procedure. Cereal Chemistry. 78(5):507–513

Anis'kov, N. I., Kalashnik, N. A., Kozlova, G. Y. A and Garis, D. V (2008). Inheritance of grain protein content upon crossing covered and hulless barley biological varieties. Russian Agricultural Science 34: 1–2

Anon, (1987). Measurement of the starch content of commercial starches. Starch 39, 414-416.

Ao, Z., Simsek, S., Zhang, G., Venkatachalam, M., Reuhs, B. L and Hamaker, B. R (2007). Starch with slow digestion property produced by altering its chain-length, branch density and crystalline structure. Journal of Agriculture and Food Chemistry *55*, 4540–4547.

Asare, E. K., Jaiswal, S., Maley, J., Båga, M., Sammynaiken, R., Rossnagel, B. G and Chibbar, R. N (2011). Barley grain constituents, starch composition, and structure affect starch in vitro enzymatic hydrolysis. Journal of Agriculture and Food Chemistry 59: 4743–4754.

Asp, N. G and Bjorck, I. (1992). Resistant starch. Trends in Food Science & Technology, 3(5), 111–114.

Atwell, W. A., Hoseney, R. C and Lineback, D. R (1980). Debraanching of wheat amylopectin . Cereal Chemistry 57: 12-21

Azhaguvel, P., Vidya-Saraswathi, D and Komatsuda, T (2006). High-resolution linkage mapping for the non-brittle rachis locus *btr1* in cultivated x wild barley (*Hordeum vulgare* L.). Plant Science 170: 1087–1094.

Badr, A., Müller, K., Schäfer-Pregl, R., El Rabey H., Effgen, S., Ibrahim, H. H., Pozzi, C., Rohde, W and Salamini F (2000). On the origin and domestication history of barley (*Hordeum vulgare*). Molecular Biology and Evolution. 17(4):499–510.

Båga M., Glaze S., Mallard C. S, Chibbar R. N (1999). A starch-branching enzyme gene in wheat produces alternatively spliced transcripts. Plant Molecular Biology 40: 1019–1030.

Båga M., Chibbar R. N and Kartha, K. K (1999). Expression and regulation of transgenes for selection of transformants and modification of traits in cereals. In:Molecular improvement of cereal crops. Vasil, K, I (ed). Kluwer Academic Press. pp83-131

Båga, M., Nair, R. B., Repellin, A., Scoles, G. J and Chibbar, R. N (2000). Isolation of a cDNA encoding a granule-bound 152-kilodalton starch-branching enzyme in wheat. Plant Physiology 124: 253–263

Båga, M., Chodaparambil, S. V., Limin, A. E., Pecar, M., Fowler, D. B and Chibbar, R. N (2007). Identification of quantitative trait loci and associated candidate genes for low-temperature tolerance in cold-hardy winter wheat. Functional & Integrative Genomics 7: 53-68

Baldwin, P (2001). Starch granule-associated proteins and polypeptides: a review. Starke. (Starch) 53, 475–503.

Ball, K and Preiss, J (1994). Allosteric sites of the large subunit of the spinach leaf ADP-glucose pyrophosphorylase. Journal of Biochemistry 269 (40): 24706-24711

Ball, S., Guan, H. P., James, M., Myers, A., Keeling, P and Mouille, G. (1996). From glycogen to amylopectin: model for the biogenesis of the plant starch granule. Cell 86: 349-352.

Ball, S., van de Wal, M. H. B. J., Visser, R. G. F (1998). Progress in understanding the synthesis of amylose. Trends Plant Science 3:462–7.

Ball, S. G and Morell, M. K (2003). From bacterial glycogen to starch: Understanding the biogenesis of the Plant Starch granule. Annual review of Plant Biology. Vol. 54: 207-233

Banks, W and Greenwood, C. T (1975). Starch and its components. Edinburgh, University Press. pp 309-325

Banks, W., Greenwood, C. T and Walker, J. T (1971). Studies on the starches of barley genotypes. A comparison of the starches from normal and high-amylose barley. *Starch/Starke*. 23, 12-15.

Baik, B. K., and Ullrich, S. E. (2008). Barley for food: Characteristics, improvement, and renewed interest. Journal of Cereal Science 48:233-242.

Bansal, H. C., Strivastava, K. N., Eggum, B. O and Mehta, S. L. (1977). Nutritional evaluation of high protein genotypes of barley. Journal Science Food and Agriculture 28(2):157-60.

Barkworth, M. E (1992). Taxonomy of the Triticeae: a hostorical perspective. Hereditas 116: 1- 14

Batey, I. L (1982). Starch analysis using thermostable alpha-amylase. Starch 34, 125-128

Batra, V. I. P., Bansal, H. C and Mehta, S. L (1982). Carbohydrate composition of developing grains of the high-lysine barley mutant (notch-2) and its parent (NP 113). Journal of Science Food and Agriculture 33: 30-34

Behall, K. M., Scholfield, D. J., Hallfrisch, J and Liljeberg-Elmstahl, H. G. M (2006). Consumption of both resistant starch and β-glucan improves postprandial plasma glucose and insulin in women. Diabetes Care 29(5):976–81.

Behall, K. M., Scholfield, D. J and Hallfrisch, J (2004). Diets containing barley significantly reduce lipids in mildly hypercholesterolemic men and women. American Journal of Clinical Nutrition. 80(5):1185-93.

Behall, K. M., Scholfield, D. J and Canary, J (1988). Effect of starch structure on glucose and insulin responses in adults. American Journal of Clinical Nutrition, 47: 428-432.

Bekele, E (1983). Some measures of gene diversity analysis on landrace populations of Ethiopian barley, Hereditas 98: 127-143.

BeMiller, J. N and Whistler, R. L (2009). Starch: Chemistry and Technology. Food Science and Technolgy International Series. BeMiller, J. N and Whistler, R. (eds). Academic Press. 30 Corporate Drive, Suite 400, Burlington , MA, 01803, USA. pp 38-389

Benmoussa, M., Moldenhauer, K. A. K and Hamaker, B. R (2007). Rice amylopectin fine structure variability affects starch digestion properties. Journal of Agriculture and Food Chemistry. 55, 1475-1479

Bergh, M. O., Razdan, A and Aman, P (1999). Nutritional influence of broiler chicken diets based on covered normal, waxy, and increased amylose barleys with or without enzyme supplementation. An. Feed Sci. Technol. 78:215-226.

Bernard, V., Brunaud, V and Lecharny, A (2010). TC-motifs at the TATA-box expected position in plant genes: a novel class of motifs involved in the transcription regulation. BMC Genomics 11:166-180

Bertoft, E., Piyachomkwan, K., Chatakanonda, P. and Sriroth, K (2008). Internal unit chain composition in amylopectins, Carbohydrate Polymers 74: 527–543.

Berry, P. M., Sterling, M and Mooney, S. J (2006). Development of a model of lodging for barley. Journal of Agronomy and Crop Science 192: 151-158.

Bhatty, R. S (1993). Non malting uses of barley. In: MacGregor A.W and Bhatty, R. S. (eds) Barley: chemistry and technology. American Association of Cereal Chemists St. Paul, MN pp 355-417.

Bhatty, R.S. (1999). "The potential of hull-less barley". Cereal Chemistry 76: 589–599

Bhatty, R. S and Rossnagel, B. G (1997). Zero amylose lines of hull-less barley. Cereal Chemistry 74:190-191.

Bhatty, R. S and Rossnagel, B. G (1998). Comparison of pearled and unpearled Canadian and Japanese barleys. Cereal Chemistry 75:15-21

Bhatty, R. S (1997). Milling of regular and waxy starch hull-less barleys for the production of bran and flour. Cereal Chemistry 74:693-699.

Bhatty, R. S (1993). Physiochemical properties of roller-milled barley bran and flour. Cereal Chemistry, v.70, p.397-402.

Bhattacharyya, M. K., Martin, C and Smith, A (1993). The importance of starch biosynthesis in the wrinkled seed shape character of peas studied by Mendel. Plant Molecular Biology 22: 525–531

Bird, A. R., Flory, C., Davies, D. A., Usher, S and Topping, D. L (2004). A novel barley cultivar (Himalaya 292) with a specific gene mutation in starch synthase IIa raises large bowel starch and short-chain fatty acids in rats. Journal of Nutrition. 134:831-835

Bjork, I., Eliasson, A-C., Drews, A., Gudmundsson, M and Karlsson, R (1990). Some nutritional properties of starch and dietary fiber in barley genotypes containing different levels of amylose. Cereal Chemistry 67: 327-333

Blakeney, A. B and Flinn, P. C (2005). Determination of non-starch polysaccharides in cereal grains with near-infrared reflectance spectroscopy. Molecular Nutrition and Food Research 49: 546-550.

Blanshard, J. M. V (1987). Starch granule structure and function: a physicochemical approach. In: Gallidard T. (ed). Starch: Properties and Potential. Chichester: John Wiley & Sons. p16-54.

Blauth, S., Yao, Y., Klucinec, J., Shannon, J., Thompson, D and Guiltinan, M (2001). Identification of *Mutator* insertional mutants of starch branching enzyme 2a (*Sbe2a*) in *Zea mays* L. Plant Physiology. 125: 1396–1405.

Blauth, S. L., Kim, K. N., Klucinec, J., Shannon, J. C., Thompson, D and Guiltinan, M (2002). Identification of Mutator insertional mutants of starch-branching enzyme 1 (sbe1) in *Zea mays* L. Plant Molecular Biology. 48: 287-297.

Bønsager, B. C., Shahpiri, A., Finnie, C and Svensson, B (2010). Proteomic and activity profiles of ascorbate–glutathione cycle enzymes in germinating barley embryo. Phytochemistry 71:1650-1656.

Borén, M., Larsson, H., Falk, A and Jansson, C (2004). The barley starch granule proteome: internalized granule polypeptides of the mature endosperm. Plant Science 166, 617–626.

Borén, M., Glaringb, M. A., Ghebremedhin, H., Olssona, H., Blennow, A and Jansson, C (2008). Molecular and physicochemical characterization of the high-amylose barley mutant Amo1. Journal of Cereal Science 47: 79–89.

Borovsky, D., Snnith, E., Whelan, W., French, D and Kikumoto, S (1979). The mechanism of Q-enzyme action and its influence on the structure of amylopectin. Archives of Biochemistry and biophysics. 198 :627-631

Bothmer von R., Jacobsen, N., Baden, C., Jørgensen R. B., and Linde-Laursen, I (eds) (1995). An ecogeographical study of the genus *Hordeum* (2nd edition). Systematic and ecogeographic studies on crop genepools 7. International Plant Genetic Resources Institute, Rome.

Bothmer, von R., Sato, K., Komatsuda, T., Yasuda, S and Fischbeck, G (2003). The domestication of cultivated barley, In: R, von Bothmer., Th. Van Hintum., H, Knuoffer and K, Sato (eds). Diversity in Barley (Hordeum vulgare), pp 9-27. Elsevier Science B.V., Amsterdam, The Netherlands.

Boyer, C. D., Garwood, D. L and Shannon, J. C (1976b). Interaction of the amylose-extender and waxy mutants of maize. Journal of Heredity 67, 209–214.

Boyer, C. D and Preiss, J (1978). Multiple forms of starch branching enzyme of maize:

evidence for independent genetic control. Biochemical and Biophysical Research Communications 80: 169-175

Boyer, C. D and Preiss, J (1981). Evidence of independent genetic control of the multiple forms of maize endosperm branching enzymes and starch synthases. Plant Physiology. 67, 1141-1145.

Bradford, M.M (1976). "Rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding", Analytical Biochemistry. 72: 248–254.

Breathnach, R and Chambon, P (1981). Organization and expression of eukaryotic split genes coding for proteins. Annu. Rev. Biochem. 50, 349–383.

Brennan, C. S (2005). Dietary fiber, glycemic responses and diabetes. Molecular Nutrition and Foods Research 49: 560- 568.

Broberg, S., Koch, K., Andersson, R and Kenne, L (2000). A comparison between MALDI-TOF mass spectrometry and HPAEC-PAD analysis of debranched starch. Carbohydrate Polymers 43 (3):285-289.

Brown, I. L (2004). Applications and uses of resistant starch. Journal of AOAC International. 87: 727–32.

Buleón, A., Colomna, P., Planchot, V and Ball, S (1998). Starch granules: structure and biosynthesis. International Journal of Biological Macromolecules. 1998, 23, 85–112.

Burkitt, D.P (1977). Food fiber: Benefits from a surgeon's perspective. Cereal Foods World 22: 6-13.

Butrim, S. M., Litvyak, V. V and Moskva, V. V (2009). A study of physicochemical properties of extruded starches of varied biological origin. Russian Journal of Applied Chemistry, Vol. 82 (7), pp. 1195−1199.

Burton, R. A., Bewley, J. D., Smith, A. M., Bhattacharyya, M. K., Tatge, H and Ring, S (1995). Starch branching enzymes belonging to distinct enzyme families are differentially expressed during pea embryo development. Plant Journal 7:3–15.

Burton, R .A., Zhang, X. Q., Hrmova, M., and Fincher, G.B (1999). A single limit dextrinase gene is expressed both in the developing endosperm and in germinated grains of barley. Plant Physiology. 119, 859–871

Burton, R. A., Johnson, P. E., Beckles, D. M., Fincher, G. B., Jenner, H. L., Naldrett, M. J and Denyer, K (2002b) Characterization of the genes encoding the cytosolic and plastidial forms of ADP-glucose pyrophosphorylase in wheat endosperm. Plant Physiology 130: 1464–1475

Burton, R. A., Jenner, H., Carrangis, L., Fahy, B., Fincher, G. B., Hylton, C., Laurie, D. A., Parker, M., Waite, D., Wegen, van S., Verhoeven, T and Denyer, K (2002). Starch granule initiation and growth are altered in barley mutants that lack isoamylase activity. Plant J. 31 97-112.

Campbell, J. A., Davies, G. J., Bulone, V and Henrissat, B (1998). A classification of nucleotide-diphospho-sugar glycosyltransferases based on amino acid sequence similarities. Biochemistry Journal 326: 929-939

Campbell, R., Mosimann, S. C., Tanner, M. E and Strynadka, N. C. J (2000). The structure of UDP-*N*-acetylglucosamine 2-epimerase reveals homology to phosphoglycosyl transferases Biochemistry. 39:14993–15001

Canadian Federation of Agriculture (1995). Agriculture in Canada. The Canadian Federation of Agriculture, Ottawa.

Canci, P. C., Nduulu, L. M., Dill-Macky, R., Muehlbauer, G. J., Rasmusson, D. C and Smith K. P (2003). Genetic relationship between kernel discoloration and grain protein concentration in barley. Crop Science. 43:1671–1679

Cai, L and Shi, Y-C (2010). Structure and digestibility of crystalline short-chain amylose from debranched waxy wheat, waxy maize, and waxy potato starches. Carbohydrate Polymers 79 : 1117–1123.

Cai, X.,Wang, Z., Xing, Y., Zhang, J. and Hong, M (1998). Aberrant splicing of intron 1 leads to the heterogeneous 5' UTR and decreased expression of waxy gene in rice cultivars of intermediate amylose content. Plant Journal. 14: 459–465.

Cao, H., Imparl-Radosevich, J., Guan, H., Keeling, P. L., James, M. G and Myers, A. M (1999). Identification of the soluble starch synthase activities of maize endosperm. Plant Physiology 120: 205–215

Ceccarelli, S and Grando, S (1991). Environment of selection and type of germplasm in barley breeding for low-yielding conditions. *Euphytica*, 57: 207-219.

Champ, M., Langkilde, A. M and Brovns, F. (2003). Advances in dietary fiber characterization 1. Definition of dietary fiber, physiological relevance, health benefits and analytical benefits. Nutrition Research Reviews, 16, 71–82.

Champ, M., Langkilde, A. M., Brovns, F., Kettlitz, B and Bail-Collet, Y. L (2003). Advances in dietary fibre characterisation. 2. Consumption, chemistry, physiology and measurement of resistant starch; implications for health and food labeling. Nutrition Research Reviews, 16, 143-161.

Chandra, G. S., Proudlove, M. O and Baxter, E. D (1999). The structure of barley endosperm—an important determinant of malt modification. Journal of the Science of Food and Agriculture 79: 37–46.

Chandrashekar, A and Mazhar, H (1999). The biochemical basis and implications of grain strength in sorghum and maize. Journal of Cereal Science 30 (3): 193–207.

Charles, A. L., Chang, Y. H., Ko, W. C., Sriroth, K and Huang, T. C (2005). Influence of amylopectin structure and amylose content on the gelling properties of five cultivars of cassava starches. Journal of Agriculture and Food Chemistry. 53, 2717–2725.

Chawla, R and Patil, G.R. (2010). Soluble Dietary Fiber. Comprehensive reviews in food science and food safety. 9 (2): 178-196

Cheng, J., Dai, F., Wei, K and Zhang G. (2007). Relationship between malt qualities and β-amylase activity and protein content as affected by timing of nitrogen fertilizer application. Zhejiang Univ Sci. B 7(1):79-84

Chevallet, M., Luche, S and Rabilloud, T (2006). Silver staining of proteins in polyacrylamide gels. Nature Protocols. 1(4): 1852–1858.

Chibbar, R. N., Ambigaipalan, P and Hoover, R (2010). Molecular diversity in pulse seed starch and complex carbohydrates and its role in human nutrition and health. Cereal Chemistry. 87(4):342–352

Chibbar, R. N and Chakraborty, M. (2005). Characteristics and uses of waxy wheat, Cereal Foods World. 50: 121–126.

Chibbar, R. N and Chakraborty, M (2005). Waxy Wheat. In Speciality grains for food and feed; Abdel-Al, E and Wood, P., eds.; American Association of Cereal Chemists, Minnesota, USA. Pp143-165

Chibbar, R. N., Ganeshan,S., Båga, M and Khandelwal, R. N (2004). Carbohydrtae metabolism. In Encyclopedia of Seed Science (1). Wringley, C., Corke, H and Walker, C (eds), Elsevier Academic Press, Oxford, pp. 168-179.

Chrastil, J (1987). Improved colorimetric determination of amylose in starches or flours. Carbohydrate Research. 159:154-158.

Chmelik, J (2001). Comparison of size characterization of barley starch granules determined by electron and optical microscopy, low angle laser light scattering and gravitational field-flow fractionation. J. Inst. Brewing, 107, 11–17.

Clark, J. R., Robertson, M and Ainsworth, C. C (1991) Nucleotide sequence of a wheat (*Triticum aestivum* L.) cDNA clone encoding the waxy protein. Plant Molecular Biology 16: 1099–1101

Clustal W: A multiple sequence alignment program for DNA or proteins. [http://www.ebi.ac.uk/Tools/clustalw/].

Colleoni, C., Myers, A. M and James, M. G (2003) One- and two-dimensional native PAGE activity gel analyses of maize endosperm proteins reveal functional interactions between specific starch metabolizing enzymes. Journal of Applied Glycoscience 50: 207–212

Colonna, P., Buleon, A and Lemarie, F (1988). Action of *Bacillus subtilis* α-amylase on native wheat starch. *Biotech. Bioeng*. 31, 895–904.

Copeland, L., Blazek, J., Salman, H and Tang, M. C (2009). Form and functionality of starch. *Food Hydrocoll.* 23: 1527–1534.

Cordain, L., Eaton, S. B., Sebastian, A., Mann, N., Lindeberg, S., Watkins, B. A., O'Keefe, J. H and Brand-Miller, J (2005). Origins and evolution of the western diet: health implications for the 21st century. American Journal of Clinical Nutrition. 81, 341–354.

Coutinho, P. M and Henrissat, B (1999). Carbohydrate-active enzymes: An integrated database approach. Recent Advances in Carbohydrate Bioengineering. Edited by: Gilbert HJ, Davies G, Henrissat B, Svensson B. Cambridge: The Royal Society of Chemistry 3-12.

Craig, J., Lloyd J.R., Tomlinson K., Barber L., Edwards A., Wang. T. L, Martin C, Hedley, C. L and Smith, A. M (1998). Mutations in the gene encoding starch synthase II profoundly alter amylopectin structure in pea embryos. Plant Cell 10:413–426.

Crawford, G. W and Gyoung-Ah, L. (2003). "Agricultural origins in the Korean Peninsula". *Antiquity* 77 (295): 87–95

Crowe, T. C., Seligman, S. A and Copeland, L (2000). Inhibition of enzymatic digestion of amylose by free fatty acids *in vitro* contributes to resistant starch formation. Journal of Nutrition. 130: 2006-2008.

Czuchajowska, Z., Sievert, D and Pomeranz, Y (1991). Enzyme-resistant starch. IV. Effects of complexing lipids. Cereal Chemistry 68(5):537–42

Dai, Z. M (2010). Activities of enzymes involved in starch synthesis in wheat (*Triticum aestivum* L.) grains differing in starch content. Russian Journal of Plant Physiology, 57(1): 74–78

Dalphin, M. E., Brown, C. M., Stockwell, P. A and Tate, W. P (1997). The translational signal database, TransTerm: more organisms, complete genomes. Nucleic Acids Research, 25 (1): 246–247

Delaney, B., Nicolosi, R. J and Wilson, T. A (2003). Beta-glucan fractions from barley and oats are similarly antiatherogenic in hypercholesterolemic Syrian golden hamsters. Journal of Nutrition 133(2):468-75

Dekker, R. F. H. and Richards, G. N (2006). Determination of starch in plant material. Journal of Science Food & Agriculture 22: 441- 444

Delrue, B., Fontaine, T., Routier, F., Decq, A., Wieruszeski, J-M., Koornhuyse, N., V. D., Maddelein, M-L., Fournet, B and Ball, S (1992). Waxy *Chlamydomonas reinhardtii*: Monocellular algal mutants defective in amylose biosynthesis and granule-bound starch synthase activity accumulate a structurally modified amylopectin. Journal of Bacteriology, 174(11): 3612-3620

Demeke, T., Hucl, P and Chibbar, R. N (1997). Immunochemical relatedness of wheat granule-bound starch synthase I (GBSSI) to other plant species. p 475. In J. L Steel and O. K Chung (ed) International Wheat Quality Conference. May 18-22, Grain Industry Alliance, Manhattan, K.S.

Demeke, T., Hucl, P., Nair, R. B., Nakamura, T and Chibbar, R. N (1997). Evaluation of canadian and other wheats for waxy proteins. Cereal Chemistry. 74(4):442–444

Demeke, T., Hucl, P., Abdel-Aal, E-S. M., Båga, M and Chibbar, R. N (1999). Biochemical characterization of the wheat waxy A proteins and its effect on starch properties. Cereal Chemistry. 76 (5): 694-698

Denyer, K., Sidebottom, C., Hylton, C. M and Smith, A. M (1993). Soluble isoforms of starch synthase and starch-branching enzyme also occur within starch granules in developing pea embryos. The Plant Journal (4): 191–198.

Denyer, K., Hylton, C. M., Jenner, C. F and Smith, A. M (1995). Identification of multiple isoforms of soluble and granule-bound starch synthase in developing wheat endosperm. Planta 196:256-265

Denyer, K., Clarke, B., Hylton, C., Tatge, H and Smith, A. M (1996). The elongation of amylose and amylopectin chains in isolated starch granules. Plant J 10: 1135–1143

Denyer, K., Waite, D., Motawia, S., Moller, B-L and Smith, A. M (1999 a) Granule-bound starch synthase I in isolated starch granules elongates malto-oligosaccharides processively. Biochemistry Journal 340: 183–191

Denyer, K., Waite, D., Edwards, A., Martin, C and Smith, A. M (1999 b) Interaction with amylopectin influences the ability of granule-bound starch synthase I to elongate malto-oligosaccharides. Biochemistry Journal 342: 647–653

Denyer, K., Johnson, P., Zeeman, S and Smith, A. M (2001). The control of amylose synthesis. Journal of Plant Physiology 158, 479–487.

Dewey, D. R (1984). The genome system of classification as a guide to hybridization with the perennial *Triticeae*. - In: Gustafson, J.P. (ed.), Gene manipulation in plant improvement. Plenum Publ. Corp., New York, pp. 209-279.

Devos, K. M (2005). Updating the 'Crop Circle'. Current opinion in plant Biology 8: 155–162.

Dinges, J. R., Colleoni, C., Myers, A. M., and James, M. G (2001). Molecular structure of three mutations at the maize *sugary1* locus and their allele-specific phenotypic effects. Plant Physiology. 125, 1406–1418.

Dinges, J. R., Colleoni, C., James, M.G and Myers, A. M (2003). Mutational analysis of the pullulanase-type debranching enzyme of maize indicates multiple functions in starch metabolism. Plant Cell 15: 666-680.

Doan D.N.P., Rudi H and Olsen O-A (1999). The allosterically unregulated isoform of ADP-glucose pyrophosphorylase from barley endosperm is the most likely source of ADP-glucose incorporated into endosperm starch. Plant Physiology, vol. 121, pp. 965–975

Domon, E., Saito, A and Takeda, K (2002a). Comparison of the waxy locus sequence from

a non-waxy strain and two waxy mutants of spontaneous and artificial origins in barley. Genes & Genetic Systems 77, 351–359.

Domon , E., Fuijita, M and Ishikawa, N (2002b). The insertion/deletion polymorphisms in the waxy gene of barley genetic resources from East Asia. Theoretical and Applied Genetics 104:132–138

Doyle, J. J and Doyle, J. L (1987). A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochemistry Bull 19: 11-15.

Drescher, A., Schreiber, H and Habekuss, A (2000). Mutations at the waxy locus of different barleys and their influence on starch synthesis. Barley Genetics VIII 3: 142–144

Dumez, S., Wattebled, F., Dauvillee, D., Delvalle, D., Planchot, V., Ball, S. G and D'Hulst C (2006). Mutants of Arabidopsis lacking starch branching enzyme II substitute plastidial starch synthesis by cytoplasmic maltose accumulation. The Plant Cell 18, 2694–2709.

Dusabenyagasani, M., Perry, D., Lewandoski, T and Demeke, T (2003). Genotyping Canadian malting barley varieties with SNP markers. Plant & Animal Genomes XI Conference. January 11-15, 2003

Eagles, H. A., Bedggood, A. G. J., Panozzo, F and Martin, P. J (1995). Cultivar and environmental effects on malting quality in barley. Australian Journal of Agricultural Research. 46:831–844

Edwards, A., Borthakur, A., Bornemann, S., Venail, J., Denyer, K., Waite, D., Fulton, D., Smith, A and Martin, C (1999a). Specificity of starch synthase isoforms from potato. European Journal of Biochemistry 266: 724-736

Edwards, A., Fulton, D. C., Hylton, C. M., Jobling, S. A., Gidley, M., Rössner, U., Martin, C and Smith, A. M (1999b). A combined reduction in activity of starch synthases II and III of potato has novel effects on the starch of tubers. The Plant Journal 17: 251–261.

Eerlingen, R. C., Jacobs, H and Delcour, J. A (1994). Enzyme-resistant starch. V. Effect of retrogradation of waxy maize starch on enzyme susceptibility. *Cereal Chemistry. 71*, 351–355.

Eliasson, A. C and Gudmundsson, M (1996). Starch: Physicochemical and functional aspects. In: Eliasson A. (ed). Carbohydrates in food. New York: Marcel Dekker, Inc. P363-449

Emanuelsson, O., Nielsen, H and Heijne, G. V (1999). ChloroP, a neural network-based method for predicting chloroplast transit peptides and their cleavage sites. Protein Science 8:978–984

Englyst, H., Wiggins, H. S and Cummings J. H (1982). Determination of the non-starch polysaccharides in plant foods by gas-liquid chromatography of constituent sugars as alditol acetates. Analyst, 107: 307-318

Englyst, H. N and Cummings, J. H (1985). Digestion of the polysaccharides of some cereal foods in the human small intestine. American Journal of Clinical Nutrition 42: 778-787.

Englyst, H. N and Macfarlane, G. T. (1986). Breakdown of resistant starch and readily digestible starch by human gut bacteria. Journal of Science Food and Agriculture 37, 699-706.

Englyst, H. N and Kingman, S. M (1990). Dietary fiber and resistant starch. A nutritional classification of plant polysaccharides. In Dietary Fiber: Chemistry, Physiology, and Health Effects; Kritchevsky, D., Bonfield, C., Anderson, J.W., Eds.; Plenum Press: New York.

Englyst, H. N., Kingsman, S. M and Cummings, J. H (1992). Classification and measurement of

nutritionally important starch fractions. European Journal of Clinical Nutrition. 46: 33S-50S

Englyst H. N and Hudson G. J. (1996). The classification and measurement of dietary carbohydrates. Food Chemisrry, 57 (1):15-21

Englyst, K. N., Englyst, H .N., Hudson, J. H., Cole, T. J and Cummings, J.H (1999). Rapidly available glucose in foods: an in vitro measurement that reflects the glycemic response. American Journal of Clinical Nutrition 69, 448–454.

Englyst, K. N and Englyst, H. N. (2005). Carbohydrate bioavailability. British Journal of Nutrition, 94, 1–11.

Erkkila, A. T., Herrington, D. M., Mozaffarian, D and Lichtenstein, A. H (2005). Cereal fiber and whole-grain intake are associated with reduced progression of coronary-artery atherosclerosis in postmenopausal women with coronary artery disease. American Heart Journal. 150(1):94-101.

Erkkila, M.J., Leah, R., Ahokas, H and Cameron-Millers, V (1998). Allells-dependant barley grain β-amylase activity. Plant Physiology, 117:679-685.

Escarpa, A., Gonzalez, M. C., Morales, M. D and Saura-Calixto, F (1997). An approach to the influence of nutrients and other food constituents on resistant starch formation. Food Chemistry 60(4):527–32.

Evans, A and Thompson, D. B (2008). Enzyme susceptibility of high-amylose starch precipitated from sodium hydroxide dispersions. Cereal Chemistry 85 (4): 480-487

Evers, A. D (1973). The size distribution among starch granules in wheat endosperm. Stärke 25, 303–304

Ezcurra, I., Ellerström, M., Wycliffe, P., Stalberg, K and Rask, L (1999). Interaction between composite elements in the *napA* promoter: both the B-box ABA-responsive complex and the RY/G complex are necessary for seed-specific expression. Plant Molecular Biology 40: 699–709.

FAO Satistical Year Book, 2008/2009. (http://www.fao.org/economic/ess/publications-studies/statistical-yearbook).

Fang, J.M., Fowler, P.A., Sayers, C and Williams, P.A (2004). The chemical modification of a range of starches under aqueous reaction conditions. Carbohydrate Polymers 55: 283–289

Fedak, G., Tsuchiya, T and Helgason, S. B (1972). Use of monotelotrisomics for linkage mapping in barley. Canadian Journal of Genetics and Cytology 14: 949–957.

Fincher, G.B. 1992. Cell wall metabolism in barley. p. 413–437. *In* P.R. Shewry (ed.) Barley: Genetics, biochemistry, molecular biology, and biotechnology. CAB Int., Wallingford, UK

Fisher, D.K., Boyer, C.D and Hannah, L.C (1993). Starch branching enzyme II from maize endosperm. Plant Physiology 102 : l045-1046.

Fitt, L. E and Snyder, E. M (1984). Photomicrographs of starches. In: Whistler R, BeMiller JN, Paschall EF. (ed). Starch chemistry and technology. 2nd ed. Orlando: Academic Press. pp 675- 682.

Florea, L., Hartzell, G., Zhang, Z., Rubin, G., and Miller, W (1998). A computer program for aligning a cDNA sequence with a senomic DNA sequence. *Genome Res.* 8: 967–974.

Forde, B. G., Heyworth, A., Pywell, J and Forde, M (1985) Nucleotide sequence of a B1 hordein gene and the identification of possible upstream regulatory elements in the storage protein genes from barley, wheat and maize. Nucleic Acids Research 13: 7327–7339

Fox, G., Kelly, A., Bowman, J., Inkerman, A., Poulsen, D and Henry, R (2009). Is malting barley better feed for cattle than feed barley? Journal of the Institute of Brewing 115(2), 95–104

Franco, C. M. L and Ciacco, C. F (1992). Factors that affect the enzymatic degradation f natural starch granules-effect of the size of the granules. Starch/Starke 44:422-26

Fregeau-Reid, J., Thin-Meiw Choo., Keh-Ming Ho., Martin, R. A and Konishi, T. (2001). Comparisons of two-row and six-row Barley for chemical composition using doubled-haploid lines. Crop Science 41 (6): 1737-1743.

French, D (1972). Fine structure of starch and its relationship to the organization of granules. Journal of the Japanese Society of starch Science 19: 8-25

French, A and Zaslow, B (1972). Conformation of the V amylose helix. Journal of Chemical. Society, Chemical Communications. 41-42.

Fu, D., Szűcs, P., Yan, L., Helguera, M., Skinner, J. S., von Zitzewitz, J., Hayes. P. M and Dubcovsky, J (2005). Large deletions within the first intron in VRN-1 are associated with spring growth habit in barley and wheat. Molecular Genetics and Genomics 273:54–65

Fujita, N., Kubo, A., Suh, D. S., Wong, K.S., Jane, J. L., Ozawa, K., Takaiwa, F., Inaba, Y and Nakamura, Y. (2003). Antisense inhibition of isoamylase alters the structure of amylopectin and the physicochemical properties of starch in rice endosperm. Plant Cell Physiology 44 : 607-618.

Fujita. N., Yoshida, M., Asakura, N., Ohdan, T., Miyao, A., Hirochika, H and Nakamura, Y (2006). Function and characterization of starch synthase I using mutants in rice. *Plant Physiology* 140(3):1070-1084

Fujita, N., Yoshida, M., Kondo, T., Saito, K., Utsumi, Y., Tokunaga, T., Nishi, A., Satoh, H., Park, J. H., Jane, J. L., Miyao, A., Hirochika, H and Nakamura, Y (2007). Characterization of SSIIIa-deficient mutants of rice: the function of SSIIIa and pleiotropic effects by SSIIIa deficiency in the rice endosperm. Plant Physiol. 144 : 2009-2023.

Fujita, N., Toyosawa, Y., Utsumi, Y., Higuchi, T., Hanashiro, I., Ikegami, A., Akuzawa, S., Yoshida, M., Mori, A., Inomata, K., Itoh, R., Miyao, A., Hirochika, H., Satoh, H and Nakamura, Y (2009). Characterization of pullulanase (PUL)-deficient mutants of rice (Oryza sativa L.) and the function of PUL on starch biosynthesis in the developing rice endosperm. Journal of Experimental Botany, 60 (3): 1009–1023.

Fulton, D. C., Edwards, A., Pilling, E., Robinson, H. L., Fahy, B., Seale, R., Kato, L., Donald, A. M., Geigenberger, P., Martin, C and Smith, A. M. (2002). Role of granule-bound starch synthase in determination of amylopectin structure and starch granule morphology in potato. Journal of Biological Chemistry, 277 (13): 10834-10841

Furukawa, K., Tagaya, M., Tanizawa, K and Fukui, T. (1993). Role of the conserved Lys-X-Gly-Gly sequence at the ADP-glucose-binding site in *Escherichia coli* glycogen synthase. Journal of Biological. Chemistry. 268, 23837–23812.

Fuwa, H., Nakajima, M., Hamada, A., & Glover, D. V. (1997). Comparative susceptibility to amylases of starches from different plant species and several single endosperm mutants and their double mutant combinations with OPAQUE-2 inbred Oh43 maize. Cereal Chemistry, 54, 230–237.

Ganeshan, S., Dahleen, L. S., Tranberg, J., Lemaux, P. G and Chibbar, R. N (2008). Barley. In: Transgenic cereals and forage grasses. Chittaranjan Kole and Timothy C. Hall (eds). pp 101-138. Blackwell Publishing Ltd.

Gao M., Fisher D. K, Kim K-N., Shannon J. C and Guiltinan M. J (1996). Evolutionary conservation and expression patterns of maize starch branching enzyme I and IIb genes suggests isoform specialization. Plant Molecular Biology 30:1223–1232

Gao, M., Wanat, J., Stinard, P. S., James, M. G and Myers, A. M (1998). Characterization of *dull1*, a maize gene coding for a novel starch synthase. Plant Cell 10: 399–412

Gao, M and Chibbar, R. N (2000). Isolation, characterization, and expression analysis of starch synthase IIa cDNA from wheat (Triticum aestivum L.). Genome 43:768–775.

Gao, J., Vasanthan1, T and Hoover, R. (2009). Isolation and characterization of high-purity starch isolates from regular, waxy, and high-amylose hulless barley grains. Cereal Chemistry 86 (2): 157-163.

Garwood, D. L., Shannon, J. C and Creech, R. G (1976). Starches of endosperms possessing different alleles at the amylose-extender locus in *Zea mays*. Cereal Chemistry 53: 355–364.

Gavel, Y and von Heijne, G (1990). A conserved cleavage-site motif in chloroplast transit peptides. FEBS Letters 261(2):455-458

Georgelis, N., Braun, E. L., Shaw, J. R and Hannah, L. C (2007). The two AGPase subunits evolve at different rates in angiosperms, yet they are equally sensitive to activity-altering amino acid changes when expressed in bacteria. Plant Cell 19: 1458-1472

Gernat, C., Krist, D., Radosta , S., Damaschun , G and Schierbaum, F (2006). Supramolecular Structure of legume starches revealed by X-ray scattering. Starch-Starke. 42 (5): 175 – 178.

Ghosh, H. P and Preiss, J (1966). Adenosine diphosphate glucose pyrophosphorylase: A regulatory enzyme in the biosynthesis of starch in spinach leaf chloroplasts. Journal of Biological Chemistry 241: 4491-4504

Gibson, T. S., Solah, V and McCleary, B. V (1997). A procedure to with concanavalin A. Journal of Cereal Science, *25*, 111–119.

Gill, S., Vasnathan, T., Ooraikul, B and Rossnagel, B (2002). Wheat bread quality as influenced by the substitution of waxy and regular barley flours in their native and extruded forms. Journal of Cereal Science. 36, 219–237.

Goňi, I., Garcia-Alonso, A and Saura-Calixto, F. (1997). A Starch hydrolysis procedure to estimate glycemic index. Nutrition Research, 17, 427-437.

Goňi, I., Garcia-Diz, L., Manas, E and Saura- Calixto, F (1996). Analysis of resistant starch: a method for foods and food products. Food Chemistry, 56 (4): 445-449,

Grabitske, H. A., & Slavin, J. L. (2009). Gastrointestinal effects of low-digestible carbohydrates. Critical Reviews in Food Science and Nutrition, 49(4), 327–360.

Griffey , C., Brooks, W., Kurantz, M., Thomason, W., Taylor, F., Obert, D., Moreau, R., Flores, R., Sohn, M and Hicks, K. (2010). Grain composition of Virginia winter barley and implications for use in feed, food, and biofuels production. Journal of Cereal Science 51: 41–49

Grimaud, F., Rogniaux, H., James, M. G., Myers, A.M and Planchot, V (2008). Proteome and phosphoproteome analysis of starch granule-associated proteins from normal maize and mutants affected in starch biosynthesis. Journal of Experimental Botany. 59 : 3395-3406.

Guan, H. P and Preiss, J (1993). Differentiation of the properties of the branching enzymes from maize (Zea mays). Plant Physiology 102: 1269 – 1273.

Gubatz, S and Shewry, P. R (2011). The development , structure and composition of the

barley grain. In: Barley: Production, improvement and uses. Ullrich, E. S (ed) pp391-438. John Wiley and Sons

Gubler, F., Li, Z., Fieg, S., Jacobsen, J. V. and Morell, M. K (2000). Cloning and characterization of a starch synthase I gene from barley. Plant Physiology. 122 (4):1459-1468

Gunkel, J., Votez, M and Rath F (2002). Effect of the malting barley variety (*Hordeum vulgare* L.) on fermentability. Journal Institute of Brewing 108:355–61.

Gupta, M., Abu-Ghannam, N., and Gallaghar, E (2010). Barley for brewing: Characteristic changes during malting, brewing and applications of its by-products. Comprehensive reviews in food science and food safety 9: 318-328

Guzmán, C. , Caballero, L., Moral , A and Alvarez, J. B (2010). Genetic variation for waxy proteins and amylose content in Spanish spelt wheat (*Triticum spelta* L.). Genetic Resources and Crop Evolution. 57(5):721-725.

Hadado, T. T., Rau, D., Bitocchi, E and Papa, R (2009). Genetic diversity of barley (*Hordeum vulgar*e L.) landraces from the central highlands of Ethiopia: comparison between the Belg and Meher growing seasons using morphological traits. Genetic Resources and Crop Evolution 56(8):1131-1148.

Hamer, H. M., Jonkers, D., Venema, K., Vanhoutvin, S., Troost, F. J and Brummer, R.-J (2010). Review article: the role of butyrate on colonic function. Alimentary Pharmacology & Therapeutics 27: 104–119

Han, X.-Z.; Ao, Z.; Janaswamy, S.; Jane, J.-L.; Chandrasekaran, R and Hamaker, B. R. (2006). Development of a low glycemic maize starch: preparation and characterization. Biomacromolecules, *7*, 1162–1168

Hanashiro, I., Tagawa, M., Shibahara, S., Iwata, K and Takeda, Y (2002). Examination of molar-based distribution of A, B and C chains of amylopectin by fluorescent labeling with 2-aminopyridine. Carbohydrate Research 337: 1211–1215

Hang, A., Don Obert, D., Gironella, A. I. N and Charlotte S. Burton, C.S (2007). Barley amylose and β-Glucan: Their relationships to protein, agronomic traits, and environmental factors. Crop Science, 47:1754-1760

Hannah, L. C (2005). Starch synthesis in the maize endosperm. Maydica 50: 497–506

Hannah, L. C and James, M (2008). The complexities of starch biosynthesis in cereal endosperms. Current Opinion in Biotechnology 19(2): 160-165

Hannah, L. C and Nelson, O. E (1976) Characterization of ADP-glucose pyrophosphorylase from *Shrunken-2* and *brittle-2* mutants of maize. Biochem Genet 14: 547–560

Haralampu, S. G. (2000). Resistant Starch-A review of the physical properties and biological impact of RS3. Carbohydrate. Polymer. 41:285-292

Harlan, J. R and Zohary, D. (1966). Distribution of wild wheats and barley. Science 153:1074–1080.

Harn, C., Knight, M., Ramakrishan, A., Guan, H., Keeling, L. P and Wasserman, P. B (1998). Isolation and characterization of the *zSSIIa* and *zSSIIb* starch synthase cDNA clones from maize endosperm. Plant Molecular Biology 37: 639-649.

Hennen-Bierwagen, T. A., Liu, F., Marsh, R. S., Kim, S., Gan, Q., Tetlow, I. J., Emes, M. J., James, M. G and Alan Myers, M (2008). Starch biosynthetic enzymes from developing maize endosperm associate in multisubunit complexes. Plant Physiology, 146:1892–1908

Henrissat, B., Coutinho, M. P and Davies, G J (2001). A census of carbohydrate-active

enzymes in the genome of *Arabidopsis thaliana.* Plant Molecular Biology 47: 55–72.
Higgins, J. A (2004). Resistant starch: metabolic effects and potential health benefits. J AOAC 87 (3): 761-768.
Hirano, H. Y Sano, Y (1991). Molecular characterization of the waxy locus of rice. Plant Cell Physiol 32:989–997.
Hirose, T and Terao, T (2004). A comprehensive expression analysis of the starch synthase gene family in rice (Oryza sativa L.). Planta 220: 9–16
Hizukuri, S., Takeda, Y and Yasuda, M. (1981). Multi-branched nature of amylose and the action of debranching enzymes. Carbohydrate Research 94:205-213.
Hizukuri S. (1985). Relationship between the distributions of the chain length of amylopectin and the crystalline structure of starch granules. Carbohydrate Research 141:295-306.
Holtekjølen, A.K., Uhlen, A.K. and Knutsen S.H. (2008). Barley carbohydrate composition varies with genetic and abiotic factors. Soil and Plant Science, 58: 27- 34
Holtekjølen, A.K., Uhlen, A.K., Bra˚then, E., Sahlstrøm, S and Knutsen, S.H (2006). Contents of starch and non-starch polysaccharides in barley varieties of different origin. Food Chemistry 94: 348–358.
Hoover, R and Sosulski, F (2006). Effect of cross-linking on functional properties of legume starches. Starch – Starke 38 (5): 149- 155.
Hoover, R. (2001). Composition, molecular structure, and physicochemical properties of tuber and root starches: A review. Carbohydrate Polymer 45:253-267
Huang, S., Elliott, R. C., Liu, P.-S., Koduri, R. K., Weickmann, J. L., Lee, J.-H., Blair, L. C., Ghosh-Dastidar, P., Bradshaw, R. A., Bryan, K. M., Einarson, B., Kendal1, R. L., Kolacz, K. H and Saito, K (1987). Specificity of cotranslational amino-terminal processing of proteins in yeast. Biochemistry 26: 8242-8246
Hucl, P and Chibbar, R. N (1996). Variation for starch concentration in spring wheat and its repeatability relative to protein concentration. Cereal Chemistry. 73, 756–758.
Hung, P. V., Maeda, T., Miskelly, D., Tsumori, R., & Morita, N. (2008). Physicochemical characteristics and fine structure of high-amylose wheat starches isolated from Australian wheat cultivars. Carbohydrate Polymers, 71, 656–663.
Hung, P. V., Maeda, T., & Morita, N. (2007). Study on physicochemical characteristics of waxy and high-amylose wheat starches in comparison with normal wheat starch. Starch/Stärke, 59, 125–131.
Hutchisonc, B (1921). Heritable characters of maize. VII. Shrunken endosperm. J. Heredity 12: 76-83.
Hylla, S., Gostner, A., and Dusel, G. (1998). Effect of resistant starch on colon in healthy volunteers: possible implication for cancer prevention. American Journal of Clinical Nutrition 67: 136-142
Hylton, C. M., Denyer, K., Keeling, P. L., Chang, M-T and Smith A. M (1996). The effect of waxy mutations on the granule-bound starch synthases of barley and maize endosperms. Planta 198:230-237
Imberty, A.. Chanzy, H., and Pérez, S. (1988). The double-helical nature of the crystalline part of A-starch, Journal of Molecular Biology 201 365-378.
Isao, H., Kensuke, O., Chieno, T and Yasuhito, T (2004). Leaching of amylose and amylopectin during cooking of rice grains and their effect on adhersiveness on of cooked rice. Journal of Applied Glycoscience 54 (4): 349-354.

Isshiki, M., Morino, K., Nakajima, M., Okagaki, R. .J, Wessler, S. R., Izawa, T and Shimamoto, K (1998) A naturally occurring functional allele of the rice waxy locus has a GT to TT mutation at the 5' splice site of the first intron. Plant J 15: 133-138.

Izydorczyk, M. S. and Dexter, J. E. (2008). Barley β-glucans and arabinoxylans: Molecular structure, physicochemical properties, and uses in food products: a review. Food Research Internation 41, *850-868.*

Izydorczyk, M.S., Storsley, J., Labossiere, D., Macgreggor, A.W and Rossnagel, B.G. (2000). Variation in total and soluble β-glucan content in hulless barley: Effects of thermal, physical and enzymic treatments. *Journal of Agriculture and Food Chemistry.* 48: 982–989

Jahan, M.S., Kumamaru, T., Hamid, A and Satoh. H (2002). Diversity of granule-bound starch synthase (GBSS) level in Bangladesh rice cultivars. Rice Genetics Newsletter. 19: 69-71.

James, M. G., Robertson, D. S and Myers, A. M (1995) Characterization of the maize gene sugary1, a determinant of starch composition in kernels. Plant Cell 7: 417–429

James, M. G., Denyer, K and Myers, A. M (2003). Starch synthesis in the cereal endosperm. Curr Opin Plant Biol 6:215–222.

Jane, J. L., Kasemsuwan, T., Leas, S., Ia, A., Zobel, H., Il, D., Robyt, J. F (1994). Anthology of starch granule morphology by scanning electron microscopy. Starch/Stärke 46, 121–129.

Jane, J. L., Shen, L., Wang, L and Maningat, C. C. (1992). Preparation and properties of small-particle cornstarch. Cereal Chemistry 69, 280–283.

Jane, J., Wong, K. S and McPherson, A. E. (1997). Branch-structure difference in starches of A- and B-type X-ray patterns revealed by their Naegali dextrins. Carbohydrate Research, 300, 219–227.

Jane, J., Chen, Y. Y., Lee, L. F., McPherson, A. E., Wong, K. S and Radosavljevic, M. (1999). Effects of amylopectin branch chain-length distributions and amylose contents on the gelatinization and pasting properties of starches. Cereal Chemistry, *76*, 629–637.

Jane, J.-L and Robyt, J. F (1984). Structure studies of amylose-V complexes and retrograded amylose by action of alpha amylases, and a new method for preparing amylodextrins. Carbohydrate Research 132: 105–118

Janthakahalli. N. V (2004) *Regulation of fructan metabolism in barley leaves.* PhD Thesis, Faculty of Science. University of Basel.

Jarvis, C. E and Walker, J. R. L (2006). Simultaneous, rapid, spectrophotometric determination of total starch, amylose and amylopectin. Journal of Science Food and Agriculture. 63 (1): 53-57.

Jayamani, P., Negrão, S., Brites, C and Oliveira, M.M (2007). Potential of Waxy gene microsatellite and single-nucleotide polymorphisms to develop japonica varieties with desired amylase levels in rice (Oryza sativa L.). Journal of Cereal Science 46: 178–186

Jenkins, D. J., Wolever, T. M and Taylor, R. H (1981). Glycemic index of foods: a physiological basis for carbohydrate exchange. American Journal of Clinical Nutrition. *34*, 362-366.

Jenkins, P. J., Cameron, R. E., Donald, A. M., Bras, W., Derbyshire, G. E., Mant, G. R and Ryan, A. J (1994). In situ simultaneous small and wide-angle X-ray scattering: a new technique to study starch gelatinization. Journal of Polymer Science *32*, 1579-1583.

Jenkins, D. J. A., Vuskan, V., Kendal, C. W. C., Wursch, P and Jeffcoat, R. (1998).

Physiological effect of resistant starches on fecal bulk, short chainfattly acids, blood lipids and glycemic index. Journal American College Nutrition. 17: 609-6115.

Jenkins, D. J. A., Axelsen, M., Kendall, C. W. C., Augustin, L. S. A., Vuksan, V and Smith, U (2000). Dietary fiber, lente carbohydrates and the insulin resistant diseases. British Journal of Nutrition. 83, 157–163.

Jeon, J-S., Ryoo, N., Hahn, T-R., Walia, H and Nakamura, Y (2010). Starch biosynthesis in cereal endosperm. Plant Physiology and Biochemistry 48 : 383-392

Jensen, M. K., Koh-Banerjee, P., Hu, F. B., Franz, M., Sampson, L., Gronbaek, M., Rimm, E. B. (2004). Intakes of whole grains, bran, and germ and the risk of coronary heart disease in men. American Journal of Clinical Nutrition 80(6):1492-9.

Jespersen, H. M., MacGregor, E. A., Henrissat, B., Sierks, M. R and Svensson, B (1993). Starch- and glycogen-debranching and branching enzymes: prediction of structural features of the catalytic $(\beta/\alpha)_8$-barrel domain and evolutionary relationship to other amylolytic enzymes. Journal of Protein Chemistry, 12 (6): 791-805.

Jobling, S. (2004). Improving starch for food and industrial applications. Current opinion in Plant Biology, 7: 1-9.

Jobling, S. A., Schwall, G. P., Westcott, R. J., Sidebottom, C. M., Debet, M., Gidley, M. J., Jeffcoat, R and Safford R (1999). A minor form of starch branching enzyme in potato (*Solanum tuberosum* L.) tubers has a major effect on starch structure: cloning and characterization of multiple forms of SBE A. The Plant Journal 18, 163–171.

Johnson, P. E., Patron, N. J., Bottrill, A. R., Dinges, J. R., Fahy, B. F., Parker, M. L., Waite, D. N and Denyer, K. (2003). A low-starch barley mutant, Risø 16, lacking the cytosolic small subunit of ADP-glucose pyrophosphorylase, reveals the importance of the cytosolic isoform and the identity of the plastidial small subunit. Plant Physiology 131: 684–696

Johnson, D., and Wilson. W. (1994). North American barley trade competition. Agricultural Economics Report No. 314, North Dakota State University.

Johnson, L. N., Acharya, K. R., Jordan, M. D and McLaughlin, P. J (1990). Refined crystal structure of the phosphorylase-heptulose 2-phosphate-oligosaccharide-AMP complex. Journal of Molecular Biology. 211, 645–661

Jood, S and Kalra, S. (2001). Chemical composition and nutritional characteristics of some hull less and hulled barley cultivars grown in India. Nahrung 45(1):35-39.

Jørgensen, K. G and Aastrup, S (1988). Quantification of high molecular weight (1→3) (1→4)-β-D-glucan using calcoflour complex formation and flow injection analysis. II Determination of total β-glucan content of barley and malt. Carlsberg Research Communique. 53: 287-296.

Joshi, C. P (1987). An inspection of the domain between putative TATA box and translation start site in 79 plant genes. Nucleic Acids Research 15 (16):6643-6653

Jia, L., Zhang, Z., Shu, X., Li, C and Wu, D. (2007). Starch properties and structure of a wheat mutant high in resistant starch. The Open Agriculture Journal, 1: 5-10

Jiang , H., Campbell, M., Blanco, M and Jane, J-L., (2010). Characterization of maize amylose-extender (ae) mutant starches: Part II. Structures and properties of starch residues remaining after enzymatic hydrolysis at boiling-water temperature. Carbohydrate Polymers 80 1–12

Jilal, A., Grando, S., Henry, R. J., Lee, L. S., Rice, N., Hill, H., Baum, M and Ceccarelli, S

(2008). Genetic diversity of ICARDA's worldwide barley landrace collection. Genetic Resources and Crop Evolution 55:1221–1230

Jui, P. Y., Choo, T. M., Ho, K. M., Konishi, T and Martin R. A. (1997). Genetic analysis of a two-row x six-row cross of barley using doubled-haploid lines. Theoretical and Applied Genetics 94: 549-556

Kandemir, N., Kudrna, D.A., Ullrich, S. E and Kleinhofs, A (2000). Molecular marker assisted genetic analysis of head shattering in six-rowed barley. Theoretical and Applied Genetics 101:203–210

Kang, H. J., Hwang, I. K., Kim, K. S and Choi, H. C (2006) Comparison of the physicochemical properties and ultrastructure of japonica and indica rice grains. Journal of Agriculture and Food Chemistry 54:4833–4838

Kanno, T., Kitano, M., Kato, R., Omori, A., Endo, Y and Tozawa, Y (2007). Sequence specifcity and efficiency of protein N-terminal methionine elimination in wheat-embryo cell-free system. Protein Expression and Purification 52:59–65

Karkalas, J. (1985). An improved enzymatic method for the determination of native and modified starch. Journal of Science, Food and Agriculture. 36: 1019-1027.

Karsai, I., Szũcs, P., Mészáros, K., Filichkina, T., Hayes, P. M and Skinner, J. S (2005). The Vrn-H2 locus is a major determinant of flowering time in facultative x winter growth habit barley (*Hordeum vulgare L*.) mapping population. Theoretical and Applied Genetics 110: 1458–1466.

Karve, M. S and Kale, N. R (1990). A spectrophotometric method for the determination of iodine binding capacity for starch and its components. Starch - Stärke 44 (1): 19-21

Kawase, M., Fukunaga, K. and Kato, K. (2005). Diverse origins of waxy foxtail barley crops in east and southeast Asia mediated by multiple transposable element insertions. Molecular Genetics and Genomics 274, 131–140.

Kim, H. S and Huber, K. C (2008). Channels within soft wheat starch A- and B-type granules. Journal of Cereal Science 48:159-172.

Kim, M and Lee, S-J (2002). Characteristics of cross-linked potato starch and starch-filled linear low-density polyethylene films. Carbohydrate Polymers 50: 331–337.

Kirby, A.R., Gunning, A.P and Morris, V.J (1996). Imaging polysaccharides by atomic force microscopy. *Biopolymers* 1996, *38*, 355-366.

Kirkman, M. A., Shewry, P. R. and Miflin, B. J. (1982) The effect of nitrogen nutrition on the lysine content and protein composition of the barley seed. Journal of Science and Agriculture. 33, 115-127.

Kislev, M.E., Nadel, D. and Carmi, I. 1992. Epipalaeolithic (19,000 BP) cereal and fruit diet at Ohalo II, Sea of Galilee, Israel. Review of Palaeobotany and Palynology 73: 161-166.

Klösgen, R. B., Gierl, A., Schwarz-Sommer, Z and Saedler, H. (1986). Molecular analysis of the waxy locus of Zea mays. Molecular General Genetics 203:237-244

Kluth, A., Sprunck, S., Becker, D., Lörz, H and Lütticke, S (2002). 5'deletion of a *gbss1* promoter region from wheat leads to changes in tissue and developmental specificities. Plant Molecular Biology 49: 669–682.

Konik-Rose, C. M., Rahman, S., Appels, R. Moss, R., McMaster, G., Marshall D. R and Stoddard, F. L (2009). Starch characterisation and variability in GBSS loci of synthetic hexaploid wheats and their durum and Aegilops tauschii parents. Euphytica 167:203–216

Kruger, J. E and Marchylo, B. A. (1985). A comparison of the catalysis of starch

components by isoenzymes from the two major groups of germinated wheat α-amylase. Cereal Chemistry, 62, 11–18.

Kubo, A. Rahman, S. Utsumi, Y. Li, Z. Mukai, Y. Yamamoto, M. Ugaki, M. Harada, K. Satoh, H. Konik-Rose, C. Morell, M.and Nakamura, Y. (2005). Complementation of sugary-1 phenotype in rice endosperm with the wheat Isoamylase1 gene supports a direct role for Isoamylase1 in amylopectin biosynthesis. Plant Physiology, 137:43–56

Kubo, A., Yuguchi, Y., Takemasa, M., Suzuki, S., Satoh, H and Kitamura, S (2008). The use of micro-beam X-ray diffraction for the characterization of starch crystal structure in rice mutant kernels of *waxy*, *amylose extender*, and *sugary1*. Journal of Cereal Science. 48 (1): 92-97

Kubo, A., Akdogan, G., Nakaya, M., Shojo, A., Suzuki, S., Satoh, H and Kitamura, S (2010). Structure, physical, and digestive properties of starch from *wx ae* double-mutant rice. Journal of Agriculture and Food Chemistry 58: 4463–4469

Kugimiya, M., Donovan, J. M and Wong, R. Y (1980). Phase transitions of amylose-lipid complexes in starches: A calorimetric study. *Starch/Stärke*, *32*: 265–270.

Kumari, S. K and Thayumanavan, B (1998). Characterization of starches of proso, foxtail, barnyard, kodo, and little millets. Plant Foods for Human Nutrition 53, 47–56.

Kwaśniewska-Karolak, I., Nebesny, E and Rosicka-Kaczmarek , J (2008). Characterization of amylose-lipid complexes derived from different wheat varieties and their susceptibility to enzymatic hydrolysis. Food Science Technology 14(1):29–37

Lafiandra, D., Sestili, F., D'Ovidio, R., Janni, M., Botticella, E., Ferrazzano, G., Silvestri, M., Ranieri, R and DeAmbrogio, E. (2010). Approaches for modification of starch composition in durum wheat. Cereal Chemistry 87(1):28–34

Larsen, M., Lund, P., Weisbjerg, M. R., Hvelplund, T. (2009). Digestion site of starch from cereals and legumes in lactating dairy cows. Animal Feed Science and Technology 153 236–248

Larson, B. L., Gilles, H. A and Jennes, R. (1953). Amperometric method for determining the sorption of iodine by starch. Analytical Chemistry, *25*(5), 802–804.

Lehmann, U and Robin, F. (2007). Slowly digestible starch- its structure and health implications: a review. Trends in Food Science and Technology, 18, 346-355.

Leterrier, M., Holappa, L. D., Broglie, K. E., and Beckles, D. M. (2008). Cloning, characterisation and comparative analysis of a starch synthase IV gene in wheat: functional and evolutionary implications. BMC Plant Biology. 8(1): 98.

Li, Z., Chu, X., Mouille, G., Yan, L., Kosar-Hashemi, B., Hey, S., Napier, J., Shewry, P., Clarke, B., Appels, R., Morell, M. K. and Rahma, S (1999). The localization and expression of the class II starch synthases of wheat. Plant Physiology, 120 :1147-1 155

Li, J. H., Vasanthan, T., Rossnagel, B., and Hoover, R. (2001a). Starch from hull-less barley: I. Granule morphology, composition and amylopectin structure. Food Chemistry. 74:395-405.

Li, Z., Sun, F., Xu, S., Chu, X., Mukai, Y., Yamamoto, M., Ali, S., Rampling, L., Kosar-Hashemi, B., Rahman, S and Morell, M. K (2003). The structural organisation of the gene encoding class II starch synthase of wheat and barley and the evolution of the genes encoding starch synthases in plants. Functional Integrated Genomics 3:76–85.

Li, J. H., Vasanthan, T., Hoover, R and Rossnagel, B. G (2004). Starch from hull-less barley: V. *In vitro* susceptibility of waxy, normal, and high amylose starches towards hydrolysis by alpha-amylases and amyloglucosidase. Food Chemistry. 84: 621–632.

Li, J., Båga, M., Rossnagel, B. C., Legge, W. G and Chibbar, R. N (2008). Identification of quantitative trait loci for β-glucan concentration in barley grain. Journal of Cereal Science. 48: 647-655

Li, Q.F., Zhang, G.Y., Dong, Z. W., Yu, H. X., Gu, M. H., Sun, S. S and Liu, Q. Q (2009). Characterization of expression of the OsPUL gene encoding a pullulanase-type debranching enzyme during seed development and germination in rice. Plant Physiology and Biochemistry. 47: 351-358

Limanskii, A. P (2007). Visualization of RNA transcripts by atomic force microscopy. Cytology and Genetic, 41(2): 76–81.

Linares, L. B., Broomhead, J. N., Guaiume, E. A., Ledoux, D. R., Veum, T. L and Raboy, V (2007). Effects of low phytate barley (*Hordeum vulgare* L.) on zinc utilization in young broiler chicks. Poultry Science 86:299–308.

Linde-Laursen, I. (1981). Giemsa banding patterns of the chromosomes of cultivated and wild barleys. - Barley Genet. IV. Proc. 4th Int. Barley Genet. Symp, Edinburgh, pp. 786-795.

Linde-Laursen, I., Bothmer, R. von & Jacobsen, N. (1986b). Giemsa C-banded karyotypes of *Hordeum secalinum, H. capense* and their interspecific hybrids with *H. vulgare*. Hereditas 105: 179-185

Lindeboom, N., Chang, P. R., and Tyler, R. P. (2004). Analytical, biochemical and physiological aspects of starch granule size, with emphasis on small granule starches: a review. Starch/ Starke, 56:89-99.

Liu, Z., Chen, S., Ouyang, Z., Guo, Y., Hu, J and M. Li (2001) Study on the chain structure of starch molecules by atomic force microscopy. Journal of Vacuum Science & Technology, B: Microelectronics and Nanometer Structures 19, 111-114.

Liu, R. H. (2004). New finding may be key to ending confusion over link between fiber, colon cancer. American Institute for Cancer Research Press Release, November 3, 2004.

Liu, J., Rong, T and Li, W (2007) Mutation loci and intragenic selection marker of the granule-bound starch synthase gene in waxy maize. Molecular Breeding 20:93–102.

Liu, F., Makhmoudova, A., Lee, E. A., Wait, R., Emes, M. J and Tetlow, I. J (2009). The amylose extender mutant of maize conditions novel protein–protein interactions between starch biosynthetic enzymes in amyloplasts. Journal of Experimental Botany, 60 (15): 4423–4440

Lloyd, J. R., Springer, F., BuleÂon, A., Mùller-Röber, B., Willmitzer, L and Kossmann, J (1999). The influence of alterations in ADP-glucose pyrophosphorylase activities on starch structure and composition in potato tubers. Planta 209: 230-238

Love, A (1984). Conspectus of the Triticeae. Feddes Report. 95:425-521

Luehrsen, K. R and Walbot, V (1991). Intron enhancement of gene expression and the splicing efficiency of introns in maize cells. Molecular Genetics 225:81–93

Lumdubwong, N and Seib, P. A. (2000). Rice starch isolation by alkaline protease digestion of wet-milled rice flour. Journal of Cereal Science. *31:* 63–74.

MacGregor, E. A (2002). Possible structure and active site residues of starch, glycogen, and sucrose synthases. Journal of Protein Chemistry, 21(4), 297-306

MacGregor, A. W and Fincher, G. F (1993). Carbohydrates of the barley grain. Barely: Chemistry and Technonoly. A. W. MacGregor and R. S. Bhatty (eds). American Association of Cereal chemistry, St. Paul, MN. Pp-73-130.

MacGregor, E. A (1993). Relationship between structure and activity in the α-amylase

family of starch-metabolising enzymes. Stärke 45 (7): 232-237
MacGregor, A. W and Balance, D.L (1980). Hydrolysis of large and small starch granules from normal and waxybarley cultivars by alpha-amylases from barley malt. Cereal Chemistry. 57: 397-402
Mahfoozi, S., Limin, A. E., Ahakpaz, F., Roustaii, M., Ketata, H and Fowler, D. B (2005). Regulation of cold tolerance in barley under field conditions in north-west Iran. Can. J. Plant Sci., 85: 597-592
Majzoobi, M., Rowe, A. J., Connock, M., Hill, S. E and Harding, S. E (2003). Partial fractionation of wheat starch amylose and amylopectin using zonal ultracentrifugation. Carbohydrate Polymer 52:269-274.
Maley J., Asare, E. K., Båga, M., Rossnagel, B. G., Chibbar, R. N and Sammynaiken, R (2010). Application of aerosol-spray deposition for determination of fine structure of barley starch using atomic force microscopy. Starch/Stärke 62: 676–685
Manners, D. J. (1989). Recent developments in our understanding of amylopectin structure. Carbohydrate Polymers, 11, 87–112.
Mangalika, W.H.A., Miura, H., Yamauchi, H and Noda, T. (2003). Properties of starches from near-isogenic wheat lines with different Wx protein deficiencies. Cereal Chemistry 80: 662–666.
Marchler-Bauer, A., Anderson, J. B., Derbyshire, M. K., DeWeese-Scott, C., Gonzales, N. R., Gwadz, M., Hao, L., He, S., Hurwitz, D. I., Jackson, J. D., Ke, Z., Krylov, D., Lanczycki, C. J., Liebert, C. A., Liu, C., Lu, F., Lu, S., Marchler, G. H., Mullokandov, M., Song, J. S., Thanki, N., Yamashita, R. A., Yin, J. J., Zhang, D and Bryant, S. H., (2007). CDD: a conserved domain database for interactive domain family analysis. Nucleic Acids Research 35: D237–D240.
Marchylo, B. A (1987). Barley cultivar identification by SDS gradient PAGE analysis of hordein. Canadian Journal Plant Science 67: 927-944.
Marconi, E., Graziano, M and Cubadda, R (2000). Composition and utilization of barley pearling by-products for making functional pastas rich in dietary fiber and β-glucans. Cereal Chemistry 77:133-139
Marti, O., Drake, B and Hansma, P.K (1987). Atomic force microscopy of liquid-covered surfaces: atomic resolution images. Applied Physics Letters. *51*: 484-486.
Marquez-Cedillo, L.A., Hayes, P.M., Kleinhofs, A., Legge, W.G., Rossnagel, B.G., Sato, K. Ullrich, S.E and Wesenberg, D.M. (2001). QTL analysis of agronomic traits in barley based on the doubled haploid progeny of two elite North American varieties representing different germplasm groups. Theoretical and Applied Genetics 103:625–637
Marszalek, P., Li, H and Fernandez, J.M (2001). Fingerprinting polysaccharides with single-molecule atomic force microscopy. Nature Biotechnology 19:258-262
Matus-Cadiz, M. A. (2000). Molecular characterization of waxy mutants in hexaploid wheat. PhD thesis. University of Saskatchewan: Saskatoon, Canada.
Matheson, N. K and Welsh, L. A. (1988). Estimation and fractionation of the essentially unbranched (amylose) and branched (amylopectin) components of starches with concanavalin A. Carbohydrate Research 180 (2): 301-313
Matthies I. E., Weise, S., Förster, J. and Röder M. S (2009). Association mapping and marker development of the candidate genes (1 -3), (1 - 4)-β-D-Glucan-4-glucanohydrolase and (1 -4)- β -Xylan-endohydrolase 1 for malting quality in barley. Euphytica 170:109-122
Mathlouthi, N., Juin H and Larbier, M (2003). Effect of xylanase and β-glucanase

supplementation of wheat- or wheat- and barley-based diets on the performance of male turkeys. British Poultry Science 44 (2): 291–298

McCleary, B. V., and Codd, R. (1991). Measurement of (1/3), (1/4)- β –Dglucan in barley and oats: A streamlined enzymic procedure. Journal of the Science of Food and Agriculture, 55, 303–312.

McCleary, B.V., Gibson,T. S. and Mugford, D. C. (1997). Measurement of total starch in cereal products by amyloglucosidase - α-amylase method: Collaborative study. Journal of AOAC Int. 80, 571-579

McCleary, B.V., Solah,V. and Gibson,T. S. (1994). Quantitative measurement of total starch in cereal flours and products. Journal of Cereal Science 20, 51-58.

McCleary, B. V and Monaghan, D. A (2002). Measurement of resistant starch. Journal of AOAC International. 85: 665-675

McDonald A.M.L., Stark J.R. (1988): A critical examination of procedures for the isolation of barley starch. Journal of the Institute of Brewing, 94: 125–132

McIntire, T.M and Brant, D.A (1999). Imaging of carrageenan macrocycles and amylose using noncontact atomic force microscopy. International Journal of Biological Macromolecules *26*, 303-310.

McKenzie, R. H., Middleton, A. B and Bremer, E. (2005). Fertilization, seeding date, and seeding rate for malting barley yield and quality in southern Alberta. Canadian Journal of Plant Science 85: 603–614.

Merritt, N.R. (1967). A new strain of barley with starch of increased amylose content. Journal of Institute of Brewers 73:583–585.

Miao, M., Jiang, B and Zhang, T (2009). Effect of pullulanase debranching and recrystallization on structure and digestibility of waxy maize starch. Carbohydrate Polymer. 76: 214-221.

Mizuno, K., Kawasaki, T., Shimada, H., Satoh, H., Kobayashi, E., Okumura, S., Arai, Y. and Baba,T. (1993). Alteration of the structural properties of starch components by the lack of an isoform of starch branching enzyme in rice seeds. Journal of Biological Chemistry. 268, 19084-19091.

Molina-Cano, J. L., Fra Mon, P., Salcedo, G., Aragoncillo, C., Roca de Togores, F and Garcia-Olmedo, F. (1987). Morocco as a possible domestication center for barley: biochemical and agromorphological evidence. Theoretical and Applied Genetics 73:531–536.

Moreau, R. A, Flores, R. A and Hicks, K. B (2007). Composition of functional lipids in hulled and hulless barley infractions obtained by scarification and in barley oil. Cereal Chemistry 84:1–5

Morera, S., Imberty, A., Aschke-Sonnenborn, U., Ruger, W and Freemont, P. S (1999). T4 Phage β-glucosyltransferase: Substrate binding and proposed catalytic mechanism. Journal of Molecular Biology 292: 717–730.

Morgan, A. G and Riggs, T. J. (1981). Effects of drought on yield and grain and malt characters in spring barley. Journal of Science Food and Agriculture. 32:339–346.

Morell, M. K., Samuel, M. S and O'Shea, M. G (1998). Analysis of starch structure using fluorophore-assisted carbohydrate electrophoresis. Electrophoresis *19:* 2603-2611

Morell, M. K. Kosar-Hashemi, B. Cmiel, M. Samuel, M. S. Chandler, P. Rahman, S. Buleon, A. Batey, I. L. Li, S. (2003). Barley *sex6* mutants lack starch synthase IIa activity and contain a starch with novel properties. Plant Journal, 34: 173-185

Morell, M.K. and Myers, A.M. (2005). Towards the rational design of cereal starches. Current. Opinion on Plant Biology. 8: 204-210.

Morrison, W. R (1993). Barley lipids. Barley: Chemistry and Technology. A. W. MacGregor and R.S. Bhatty (eds). American Association of Cereal Chemists, St. Paul, MN.

Morrison, W.R and Laignelet, B. (1983). An improved colorimetric procedure for determining apparent and total amylose in cereal and other starches. Journal of Cereal Science. 1:9-20

Mouille, G., Maddelein, M.-L., Libessart, N., Talaga, P., Decq, A., Delrue, B and Ball, S. (1996). Phytoglycogen processing: A mandatory step for starch biosynthesis in plants. Plant Cell 8, 1353–1366.

Mussatto, S. I., Dragone, G and Roberto, I. C (2006). Brewers' spent grain: generation, characteristics and potential application. Journal of Cereal Science 43:1–14.

Mua, J. P and Jackson, D. S (1995). Fractionation of regular corn starch: Comparison of aqueous leaching and aqueous dispersion method. Cereal Chemistry. 72: 508-511

Muir JG, Birkett A, Brown, I, Jones G and O'Dea K (1995). Food processing and maize variety affects amounts of starch escaping digestion in the small intestine. American Journal of Clinical Nutrition 61, 82–89.

Murai, J., Taira, T and Ohta, D. (1999). Isolation and characterization of the three waxy genes encoding the granule-bound starch synthase in hexaploid wheat. Gene 234:71-79

Mussatto, S. I., Dragone, G and Roberto, I. C (2006). Brewers' spent grain: generation, characteristics and potential application. Journal of Cereal Science 43:1–14.

Mutisya, J., Sun, C., Palmqvist, S., Baguma, Y., Odhiambo, B and Jansson, C (2006). Transcriptional regulation of the *sbeIIb* genes in sorghum (*Sorghum bicolor*) and barley (*Hordeum vulgare*): Importance of the barley sbeIIb second intron. Journal of Plant Physiology 163: 770—780

Myers A.M, Morell, M.K, James MG, Ball S. (2000). Recent progress toward understanding biosynthesis of the amylopectin crystal. Plant Physiology 122:989–998.

Nair, R. B., Båga, M., Scolesb, G. J., Kartha, K. K., Chibbar, R. N (1997). Isolation, characterization and expression analysis of a starch branching enzyme II cDNA from wheat. Plant Science 122: 153- 163

Nakamura, Y and Yamanouchi, H. (1992). Nucleotide sequence of a cDNA encoding starch-branching enzyme, or Q-enzyme I, from rice endosperm. Plant Physiology. 99(3): 1265–1269.

Nakamura, T., Yamamori, M., Hirano, H and Hidaka, S (1993). The waxy (Wx) proteins of maize, rice and barley. *Phytochemistry* 33:749–753

Nakamura, Y., Umemoto, T., Ogata, N., Kuboki, Y., Yano, M and Sasaki, T. (1996). Starch debranching enzyme (R-enzyme or pullulanase) from developing rice endosperm: purification, cDNA and chromosomal localization of the gene. Planta 199: 209-218

Nakamura, Y., Umemoto, T., Takahata, Y., Komae, K., Amano, E and Satoh, H (1996). Changes in structure of starch and enzyme activities affected by *sugary* mutations in developing rice endosperm. Possible role of starch debranching enzyme (R-enzyme) in amylopectin biosynthesis. Physiologia Plantarum 97: 491-498.

Nakamura, T., Vrinten, P., Hayakawa, K and Ikeda, J (1998) Characterization of a granule-bound starch synthase isoform found in the pericarp of wheat. Plant Physiology 118: 451–459

Nakamura Y. (2002). Towards a better understanding of the metabolic system for

amylopectin biosynthesis in plants: rice endosperm as a model tissue. Plant Cell Physiology 43:718–25.

Nakamura, Y., Sakurai, A., Inaba, Y., Kimura, K., Iwasawa, N and Nagamine, T (2002). The fine structure of amylopectin in endosperm from Asian cultivated rice can be largely classified into two classes Starch 54:117 131.

Nakamura, Y., Francisco, P. B. Jr., Hosaka, Y., Satoh, A., Sawada, T., Kubo, A and Fujita, N (2005) Essential amino acids of starch synthase IIa differentiate amylopectin structure and starch quality between japonica and indica rice varieties. Plant Molecular Biology 58: 213–227

Nedeltscheva , M., Stoilkov , G., Popova, S. (1975). A modified analysis method of starch determination by iodine spectrophotometry. Starch-Stärke 27 (9): 298 - 301

Newman, C. W and McGuire. C. F (1985). Nutritional quality of barley. In: Rasmusson, C. D (eds.) Barley. American Society of Agronomy, Madison, Wisconsin pp 403-456

Newman, C. W and Newman, R. K (2006). A brief history of barley foods. Cereal Foods World 51: 4–7.

Newman, C. W and Newman, R. K (2005). Huless barley for food and feed. Speciality garins for food and feed. Abdel-Al, E and Wood, P. J (eds). American Association of Cereal Chemistry, St Paul, MN

Newman, C. W and Newman. R. K. (1992). Nutritional aspects of barley seed structure and composition. p. 351–368. *In* P.R. Shewry (ed.) Barley: Genetics, biochemistry, molecular biology, and biotechnology. CAB Int., Wallingford, UK.

Nghiem, N. P., Hicks, K. B., Johnston, D. B., Senske, G., Kurantz, M., Li, M., Shetty, J and Konieczny-Janda, G (2010). Production of ethanol from winter barley by the EDGE (enhanced dry grind enzymatic) process. Biotechnology for Biofuels 3:8-15

Niba, L. L (2002). Resistant starch: a potential functional food ingredient. Nutrition and Food Science 32 (2): 62-67

Nichols, D. J., Keeling, P. L., Spalding, M and Guan, H (2000). Involvement of conserved aspartate and glutamate residues in the catalysis and substrate binding of maize starch synthase. Biochemistry. 39, 7820 –7825.

Nielsen, M. M., Bozonnet, S., Seo, E-S., Mótyán, J. A., Andersen, J. M., Dilokpimol, A., Hachem, M. A., Gyémánt, G., Næsted, H., Kandra, L., Sigurskjold, B. W and Svensson, B (2009). Two secondary carbohydrate binding sites on the surface of barley r-amylase 1 have distinct functions and display synergy in hydrolysis of starch granule. Biochemistry 48: 7686–7697.

Nilan, R.A. and Ullrich, S.E. (1993). Barley: Taxonomy, origin, distribution, production, genetics, and breeding. *In* W. MacGregor and R.S. Bhatty (eds.) Barley chemistry and technology. American Association of Cereal Chemists, Inc., St. Paul, MN.
Perspectives on new crops and new uses. ASHS Press, Alexandria, VA.

Nishi, A., Nakamura, Y., Tanaka, N and Satoh, H (2001). Biochemical and genetic analysis of the effects of amylose-extender mutation in rice endosperm. Plant Physiol. 127 459-472.

Noda, T., Takahata, Y., Sato, T., Suda, I., Morishita, T., Ishiguro, K and Yamakawa, O (1998). Relationships between chain length distribution of amylopectin and gelatinization properties within the same botanical origin for sweet potato and buckwheat. Carbohydrate Polymers. 32 (2): 153-158

North Dakota Agricutural Statistics Services (1997). Online service: http//www.usda.gov.nass.nd

Notredame, C., Higgins, D. G. and Heringa, J (2000). T-Coffee: A novel method for fast and accurate multiple sequence alignment. Journal of Molecular Biology 302: 205-217

O'Dea, K., Nestel, P. J and Antonoff, L (1980). Physical factors influencing postprandial glucose and insulin responses to starch. American Journal of Clinical Nutrition 33: 760-765.

O'Dea, K., Snow, P and Nestel, P (1991). Rate of starch hydrolysis in vitro as a predictor of metabolic responses to complex carbohydrate in vivo. American Journal of Clinical Nutrition 34 1991-1993.

Ogushi, K., Lim, P., Barr, A.R., Takahashi, S., Asakura, T and Ito, K (2002). Japanese barley meets Australia: quality performance of malting barley grown in different countries. Journal of the Institute of Brewing 108, 303–309.

Ohdan, T., Francisco P.B., Sawada, T., Hirose, T., Terao, T., Satoh, H and Nakamura, Y. (2005). Expression profiling of genes involved in starch synthesis in sink and source organs of rice. Journal of Experimental Botany 56: 3229-3244.

Okita, T. W (1992). Is there an alternative pathway for starch synthesis? Plant Physiology. 100: 560-564

Oscarsson, M., Parkkonen, T., Autio, K., and Åman, P. (1997). Composition and microstructure of normal, waxy and high-amylose barley samples. Journal of Cereal Science 26:259-264.

Oscarssson, M., Andersson, R., Solomonsson, A. C and Aman, P (1996). Chemical composition of barley samples focusing in dietary fiber components. Journal of Cereal Science 24: 161-170.

O'Shea, M. G., Samuel, M. S., Konik, C. M and Morell, M. K (1998). Fluorophore-assisted carbohydrate electrophoresis (face) of oligosaccharides: efficiency of labelling and high-resolution separation. Carbohydrate Research 307:1-12.

O'Shea, M. G and Morell, M. K (1996). High resolution slab gel electrophoresis of 8-amino-1, 3, 6-pyrenetrisulfonic acid (ATPS) lagged oligosaccharides using a DNA sequencer. Electrophoresis 17: 681-688

Palmer, G.H (2000). Malt performance is more related to in-homogeneity of protein and beta glucan breakdown than to standard malt analyses. Journal of the Institute of Brewing 106 (3), 189–192.

Palmer, G. H., and Mackenzie, C. I. 1986. Levels of alkali-soluble β-D-glucans in cereal grains. Journal of the Institute of Brewing 92:461-462.

Pan, D and Nelson, O. E (1984) A debranching enzyme deficiency in endosperms of the sugary-1 mutants of maize. Plant Physiology 74: 324-328

Patron N. J., Smith A. M., Fahy B. F., Hylton C. M., Naldrett M. J., Rossnagel B. G. and Denyer K. (2002). The Altered Pattern of Amylose Accumulation in the Endosperm of Low-Amylose Barley Cultivars Is Attributable to a Single Mutant Allele of Granule-Bound Starch Synthase I with a Deletion in the 5'-Non-Coding Region. Plant Physiology 130: 190-198.

Paz-Ares, J., Hernandez-Lucas, C., Salcedo, G., Aragoncillo, C., Ponz, F and Garcia-Olmedo, F (1982). The CM-proteins from cereal endosperm: immunochemical relationships. Journal of Experimental Botany. 34(141): 388-395.

Pedersen, J. F., Graybosch, R. A and Funnell, D. L (2007). Occurrence of the waxy alleles wx^a and wx^b in waxy sorghum plant introductions and their effect on starch thermal properties. Crop Science 47:1927–1933.

Pedersen, J. F., Bean, S. R., Graybosch, R. A., Park, S. H and Tilley, M (2005).

Characterization of waxy grain sorghum lines in relation to granule-bound starch synthase. Euphytica 144: 151–156

Peng, M., Gao, M., Båga, M., Hucl, P and Chibbar, R. N (2000). Starch-branching enzymes preferentially associated with a-type starch granules in wheat endosperm. Plant Physiology, 124: 265–272

Peng, M.; Gao, M; Abdel-Aal, E. S. M.; Hucl, P.; Chibbar, R. N. (1999). Separation and characterization of A- and B-type starch granules in wheat endosperm. Cereal Chemistry 76: 375–379

Perveen, A., Naqvi, I. I., Shah, R. and Hasnain, A. (2008). Comparative germination of barley seeds (hordeum vulgare) soaked in alkaline media and effects on starch and soluble proteins. Journal of Applied Science and Environment 12 (3) 5-9

Planchot, V., Colonna, P., Buleon, A and Gallent, D. (1997). Amylolysis of starch granules and α-glucan crystallites. In R. J. Frazier, A. M. Donald, & P. Richmond (Eds.), Starch structure and functionality (pp. 141–152). Cambridge: Royal Society of Chemistry, 141–152.

Pourkheirandish, M and Komatsuda, T (2007). The importance of barley genetics and domestication in a global perspective. Annals of Botany 100: 999–1008.

Powell, W. Ellis' R. P and Thomas, W. T. B. (1990). The effects of major genes on quantitatively varying characters in barley. Ill. The two row/six row locus (V—v). Heredity 65 : 259-264

Powell, W., Caliagari, P. D. S., Swanston, J. S and Jinks, J. L (1985). Genetic investigations into β-glucan content in barley. Theoretical and Applied Genetics 71, 461–466.

Preiss, J. (1988). Biosynthesis of starch and its degradation. In: The Biochemistry of Plants, Vol 14, pp181-254. Academic Press. San Diego

Preiss, J., Danner, S., Summers, P. S., Moreill, M., Barton, C. R., Yang, L and Nieder, M (1990). Molecular characterization of the brittle-2 gene effect on maize endosperm ADPglucose pyrophosphorylase subunits Plant Physiology 92: 881-885.

Preiss, J and Sivak, M (1996) Starch synthesis in sinks and sources. In E Zamski, AA Schaffer, eds, Photoassimilate Distribution in Plants and Crops. Marcel Dekker, New York, pp 63–96

Prosky, L., Asp, N.G., Schweizer, T. F., DeVries, J. W and Furda, I (1988). Determination of insoluble, soluble, and total dietary fiber in foods and food products: inter laboratory study. J. *AOAC*. 71, 5, 1017-1023.

Psota, V., Vejrazka, K., Famera, O and Hrcka, M. (2007). Relationship between grain hardness and malting quality of barley (*Hordeum vulgare* L.). J Inst Brew 113:80–86.

Puchongkavarin,H.,Varavinit,S and Bergthaller,W.(2005).Comparative study of pilot scale rice starch production by an alkaline and an enzymatic process. Starch/Stärke, 57, 134–144.

Putseys, J. A., Derde, L. J., Lamberts, L., Ostman, E., Bjrck, I. M and Delcour, J. A (2010). Functionality of short chain amylose−lipid complexes in starch−water systems and their impact on *in vitro* starch degradation. Journal of Agriculture Food and Chemistry 58, 1939-1945

Qian, J., Jiang, S., Su, W and Gao, P (2009). Characteristics of oil from hulless barley (Hordeum vulgare L.) bran from Tibet. Journal of American Oil Chemical Society 86:1175–1179

Radchuk, V. V., Borisjuk, L., Sreenivasulu, N., Merx, K., Mock, H. P., Rolletschek, H., Wobus, U and Weschke, W (2009). Spatio-temporal profiling of starch biosynthesis and degradation in the developing barley grain. Plant Physiology 150, 190–204.

Rahman, S., Kosarhashemi, B., Samuel, M. S., Hill, A., Abbott, D. C., Skerritt, J. H., Preiss, J., Appels, R and Morell, M. K (1995). The major proteins of wheat endosperm starch granules. Australian Journal of Plant Physiology 22: 793–803.

Rahman, S., Regina, A., Li Z., Mukai Y., Yamamoto M., Hashemi B. K., Abrahams S and Morell M. K (2001) Comparison of starch branching enzyme genes reveals evolutionary relationships among isoforms. Characterisation of a gene for starch branching enzyme IIa from the wheat D genome donor Aegilops tauschii. Plant Physiology 125:1314–1324

Ral, J. P., Colleoni, C.,Wattebled, F., Dauvillee, D., Nempont, C., Deschamps, P., Li, Z., Morell, M. K., Chibbar, R., Purton, S., d'Hulst, C and Ball, S. G (2006) Circadian clock regulation of starch metabolism establishes GBSSI as a major contributor to amylopectin synthesis in Chlamydomonas reinhardtii. Plant Physiol 142: 305–317

Rastogi, V and Oaks, A (1986).Hydrolysis of storage proteins in barley endosperm. Plant Physiology.81: 901-906

Ratnayake, W. S and Jackson, D. S. (2008). Thermal behaviour of resistant starches RS 2, RS 3, and RS 4. Journal of Food Science 73(5): 356–366.

Regina, A., Kosar-Hashemi, B., Li, Z., Rampling, L., Cmiel, M., Gianibell, C., Konik-Rose, C., Larroque, O., Rahman, S and Morell, M. K (2004). Multiple isoforms of starch branching enzyme I in wheat: lack of the major SBE-I isoform does not alter starch phenotype. Functional Plant Biology 31, 591–601.

Regina, A., Kosar-Hashemi, B., Li, Z., Pedler, A., Mukai, Y., Yamamoto, M., Gale, K., Sharp, P. J., Morell, M. K and Rahman, S (2005). Starch branching enzyme IIb in wheat is expressed at low levels in the endosperm compared to other cereals and encoded at a non-syntenic locus. Planta 222: 899–909

Regina, A., Bird, A., Topping, D., Bowden, S., Freeman, J., Barsby, T., Kosar-Hashemi, B., Li, Z., Rahman, S and Morell, M (2006). High-amylose wheat generated by RNA interference improves indices of large-bowel health in rats. PNAS (10): 3546–3551

Regina, A., Kosar-Hashemi, B., Ling, S., Li1, Z., Rahman, S and Morell, M (2010). Control of starch branching in barley defined through differential RNAi suppression of starch branching enzyme IIa and IIb. Journal of Experimental Botany 61 (5):1469–1482.

Repellin A, Båga M, and Chibbar R. N (2008). *In vitro* pullulanase activity of wheat (Triticum aestivum L.) limit-dextrinase type starch debranching enzyme is modulated by redox conditions. J.of Cereal Science. 47:302-309

Repellin A, Båga M, and Chibbar R. N (2001). Characterization of a cDNA encoding a type I starch branching enzyme produced in developing wheat (*Triticum aestivum* L.) kernels. Journal of Plant Physiology 158: 91-100

Roberfroid, M. B. (2000). Prebiotics and probiotics: Are they functional foods? American Journal of clinical Nutrition, 71(6), 1682–1687.

Robin, J. P., Mercier, C., Charbonniere, R. & Guilbot, A. (1974). Lintnerizes starches: Gel filtration and enzymatic studies of insoluble residues from prolonged treatment of potato starch. Cereal Chemistry 51:389-406.

Rohde, W., Becker, D and Salamini, F (1988). Structural analysis of the waxy locus from *Hordeum vulgare*. Nucleic Acids Research 16 (14): 1785- 1786

Roldan, I., Wattebled, F., Lucas, M. M., Delvalle, D., Planchot, V., Jimenez, S., Perez, R.,

Ball, S., D'Hulst, C and Merida, A (2007). The phenotype of soluble starch synthase IV defective mutants of *Arabidopsis thaliana* suggests a novel function of elongation enzymes in the control of starch granule formation. Plant Journal, 49(3):492-504.

Rosin, P. M., Lajolo, F. M and Menezes, E. W (2002). Measurement and characterization of dietary starch. Journal of Food Composition and Analysis. 15:367–377.

Roslinsky, V., Eckstein, P. E., Raboy, V., Rossnagel, B. G and Scoles, G. J (2007). Molecular marker development and linkage analysis in three low phytic acid barley (Hordeum vulgare) mutant lines. Molecular Breeding 20:323–330

Rozen,S and Skaletsky H. J. (2000) In: Krawetz S, Misener S (eds) *Bioinformatics Methods and Protocols: Methods in Molecular Biology.* Humana Press, Totowa, NJ, pp 365-386

Russell, P. L., Berry, C. S and Greenwell, P (1989). Characterisation of resistant starch from wheat and maize. Journal of Cereal Science. 9:1–15.

Sajilata, M. G., Singhal, R. S and Kulkarni, P. R (2006). Resistant starch-a review. Comprehensive reviews in food science and food safety. 5: 1-17.

Sakulsingharoja, C., Choi, S. B., Hwang, S. K., Edwards, G. E., Bork, J., Meyer, C. R., Preiss, J and Okita, T. W(2004) Engineering starch biosynthesis for increasing rice seed weight: the role of the cytoplasmic ADP-glucose pyrophosphorylase. Plant Science 167: 1323–1333

Salcedo, G., P. Fra-Mon, J.L. Molina-Cano, C. Aragoncillo and F. García-Olmedo (1984). Genetics of CM-proteins (A-hordeins) in barley. Theoretical and Applied Genetics. 68, 53-59

Salman, H., Blazek, J., Lopez-Rubio, A., Gilbert, E. P., Hanley T and Copeland, L (2009). Structure–function relationships in A and B granules from wheat starches of similar amylose content. Carbohydrate Polymers 75: 420–427

Salomonsson, A and Sundberg, B (1994). Amylose content and chain profile of amylopectin from normal, increased amylose and waxy barleys. Starch/Staerke, 46, 325–328.

Sambrook, J., Fritsch, E. F and Maniatis, T (1989). Molecular Cloning: a laboratory manual. 2nd ed. N.Y., Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press, 1659 p

Sargeant, J.G (1982). Determination of amylose:amylopectin ratios of starches. Starch 34, 89–92

Sato, Y and Nishio, T (2003). Mutation detection in rice waxy mutants by PCR-RF-SSCP. Theor Appl Genet 107:560–567.

Satoh, H., Shibahara, K., Tokunaga, T., Nishi, A., Tasaki, M., Hwang, S.K., Okita, T.W., Kaneko, N., Fujita, N., Yoshida, M., Hosaka, Y., Sato, A., Utsumi, Y., Ohdan, T and Nakamura, Y (2008). Mutation of the plastidial alpha-glucan phosphorylase gene in rice affects the synthesis and structure of starch in the endosperm. Plant Cell 20: 1833-1849.

`

Satoh, H., Nishi, A., Yamashita, K., Takemoto, Y., Tanaka, Y., Hosaka, Y., Sakurai, A., Fujita, N., Nakamura, Y (2003). Starch-branching enzyme I-deficient mutation specifically affects the structure and properties of starch in rice endosperm. Plant Physiolgy. 133 1111-1121.

Satoh, H., Nishi, A., Fujita, N., Kubo, A., Nakamura, Y and Kawasaki, T (2003a). Isolation and characterization of starch mutants in rice. Journal of Applied Glycoscience. 50 : 225 – 230

Schmidt, R. J., Ketudat, M., Aukerman, M. J and Hoschek, G. (1992). Opaque-2 is a

transcriptional activator that recognizes a specific target site in 22-kD zein genes. Plant Cell 4: 689–700.

Schmitt, M. R and Wise, M. L (2009). Barley and oat β-glucan content measured by calcofluor fluorescence (CF) in a microplate assay. Cereal Chemistry. 86(2):187–190

Schmitz, A and Gray, R. (2000). "The Canadian Wheat Board and Feed Barley", Agribusiness Vol 16, #4.Schupp, N and Ziegler, P (2004). The Relation of Starch Phosphorylases to Starch Metabolism in Wheat. Plant Cell Physiology 45(10): 1471–1484

Schondelmaier, J., Jacobi, A., Fischbeck, G and Jahoor. A (1992). Genetical studies on the mode of inheritance and localization of the amo1 (high amylose) gene in barley. Plant Breeding 109:274-280

Schwall, G. P., Safford, R., Westcott, R..J., Jeffcoat, R., Tayal, A., Shi, Y. C., Gidley, M. J and Jobling, S. A (2000). Production of very increased amylose potato starch by inhibition of SBE A and B. Nat Biotechnol. 18, 551-554.

Seal, C. J., Daly, M. E., Thomas, L. C., Bal, W., Birkett, A., Jeffcoat M and R., Mathers, J. C (2003). Postprandal carbohydrate metabolism in healthy subject and those with type 2 diabetes fed starches with slow and rapid hydolysis rates determined *invitro*. British Journal of Nutrition 90: 853-864

Senoura, T., Asao, A., Takashima, Y., Isono, N., Hamada, S., Ito, H., and Matsui, H. (2007). Enzymatic characterization of starch synthase III from kidney bean (*Phaseolus vulgaris* L.). FEBS Journal 274(17): 4550–4560.

Sestili, F.,Botticella, E., Bedo, Z., Phillips, A and Lafiandra, D. (2010). Production of novel allelic variation for genes involved in starch biosynthesis through mutagenesis. Molecular Breeding 25:145–154

Shapter, F.M., Eggler, P., Lee, L.S and Henry, R.J (2009). Variation in Granule Bound Starch Synthase I (GBSSI) loci amongst Australian wild cereal relatives (Poaceae). Journal of Cereal Science 49: 4–11.

Sharma, A., Yadav, B. S and Ritika, S (2008). Resistant starch: Physiological roles and food applications. Food Reviews International, 24, 193–234.

Shewry, P. R. Faulks, A. J., Pickering, R. A., Jones, I. T., Finch, R. A and Miflin, B. J. (1980). The genetic analysis of barley storage proteins. Heredity 44: 383-889

Shewry, P. R and Parmer, S (1987) The HrdF (Hor5) locus encodes nu-type hordeins. Barley Genet Newslett 17: 32-35

Shewry, P.R. (1993). Barley seeds proteins. In: Macgregor, A.W.; Bhatty, R.S. Barley: Chemistry and technology. St. Paul: AACC p.1-5.

Shewry, P. R and Halford, N. G (2002). Cereal seed storage proteins: structures, properties and role in grain utilization. Journal of Experimental Botany, 53(370): 947–958

Shi, X and BeMiller, J. N (2002). Aqueous leaching of derivatized amylose from hydroxypropylated common Corn starch granules. Starch - Stärke 54 (1): 16-19

Shi , Y-C and Seib, P. A. (1995). Fine structure of maize starches from four *wx*- containing genotypes of the W64A inbred line in relation to gelatinization and retrogradation. Carbohydrate Polymers 26: 141-147.

Shu, X. L., Shen, S. Q., Bao, J. S., Wu, D. X., Nakamura, Y and Shu, Q. Y (2006). Molecular and biochemical analysis of the gelatinization temperature characteristics of rice (*Oryza sati*V*a* L.) starch granules. Journal of Cereal Science *44:* 40–48.

Shure, M., Wessler, S and Fedoroff, N (1983). Molecular identification and isolation of the waxy locus in maize. Cell, 35, 225 -233

Sikka, V. K., Choi, S., Kavakl, I. H., Sakulsingharoj C., Gupta S., Ito H., Okita, T. W (2001) Subcellular compartmentation and allosteric regulation of the rice endosperm ADPglucose pyrophosphorylase. Plant Science 161: 461–468

Singh, S., Singh, N., Isono, N and Noda, T. (2010). Relationship of granule size distribution and amylopectin structure with pasting, thermal, and retrogradation properties in wheat starch. Journal of Agriculture and Food Chemistry 58: 1180–1188

Sissons, M. J., Lance, R. C. M and Sparrow, D. H. B (1993) Studies on Limit Dextrinase in Barley. 3. Limit Dextrinase in Developing Kernels. Journal of Cereal Science. 17, 19–24.

Small, E. (1999). New crops for Canadian agriculture. p. 15–52. In: J. Janick (ed.), Smidansky E. D, Martin J. M, Hannah L. C, Fischer A. M, Giroux MJ (2003). Seed yield and plant biomass increases in rice are conferred by deregulation of endosperm ADP-glucose pyrophosphorylase. Planta 6: 656–664

Smidansky, E. D, Clancy, M., Meyer, F. D., Lanning, S. P, Blake, N. K., Talbert, L. E and Giroux, M. J (2002) Enhanced ADP-glucose pyrophosphorylase activity in wheat endosperm increases seed yield. Proceedings of National Academy of Science USA 99: 1724–1729

Smidansky, E. D., Martin, J. M., Hannah, L. C., Fischer, A. M. and Giroux, M. J (2003). Seed yield and plant biomass increases in rice are conferred by deregulation of endosperm ADP-glucose pyrophosphorylase. *Planta*, 216: 656–664.

Smith A. M. (2001). The biosynthesis of the starch granule. Biomacromolecules. 2:335– 41

Smith, K. N., Queenan, K. M., Thomas, W. R., Fulcher, G and Slavin, J. L (2008). Physiological effects of concentrated barley –glucan in mildly hypercholesterolemic adults. Journal of American College of nutrition 27 (3): 434-440

Smith-White B. J and Preiss J. (1992). Comparison of proteins of ADP-glucose pyrophosphorylase from diverse sources. Journal of Molecular Evolution 34: 449–464

Smith, D. B., Gill, A. A and Ahluwalia, B. (1987). Cultivar differences in the concentration of β-D glucans in developing and mature barley grains and their significance for malting quality. Cereal Quality. Asp. App. Bio 15: 105-113.

Song, Y.and Jane J. (2000). Characterization of barley starches of waxy, normal, and high amylose varieties. Carbohydrate Polymers 41: 365-377

Sopanen, T.and Lauriere, C (1989). Release and activity of bound, β-amylase in a germinating barley grain. Plant Physiol. 89:244-249.

South J.B and Morrison W.R. (1990): Isolation and analysis of starch from single kernels of wheat and barley. Journal of Cereal Science, 12: 43–51.

Sparks, M. E and Brendel, V (2005). Incorporation of splice site probability models for non-canonical introns improve gene structure prediction in plants. Bioinformatics 21(3):20-30

Sparks, M. (2005). The Canadian barley industry in transition: A Study for Alberta Agriculture Food and Rural Development.

Sreenivasulu, N., Usadel, B., Winter, A., Radchuk, V., Scholz, U., Stein, N., Weschke, W., Strickert, M., Close, T. J., Stitt, M., Graner, A and Wobus, U (2008). barley grain maturation and germination: Metabolic pathway and regulatory network commonalities and differences highlighted by new MapMan/PageMan profiling tools. Plant Physiology, 146: 1738–1758

Srichuwong, S., Sunarti, T. C., Mishima, T., Isono, N and Hisamatsu, M (2005). Starches from different botanical sources I: Contribution of amylopectin fine structure to thermal properties and enzyme digestibility. Carbohydrate Polymers 60: 529–538.

Stahl, Y., Coates, S., Bryce, J. H and Morris, P. C (2004). Antisense downregulation of the barley limit dextrinase inhibitor modulates starch granule size distribution, starch composition and amylopectin structure. The Plant Journal 39: 599–611

Stark, D, Timmerman K, Barry G, Preiss J, Kishore G (1992). Regulation of the amount of starch in plant tissues by ADP glucose pyrophosphorylase. Science 258: 287–291

Stevnebø, A., Sahlström, S and Svihus, B (2006). Starch structure and degree of starch hydrolysis of small and large starch granules from barley varieties with varying amylose content. Animal Feed Science and Technology 130, 23–38.

Sulaiman B.D., Morrison W.R. (1990): Proteins associated with the surface of wheat starch granules purified by centrifuging through caesium chloride. Journal of Cereal Science, 12: 53–61.

Suortti , T., Gorenstein , M.V and Roger, P (1998). Determination of the molecular mass of amylose, Journal of Chromatography *A* 828 pp. 515–521.

Sun C, Sathish P, Ahlandsberg S and Jansson C (1998). The two genes encoding starch-branching enzymes IIa and IIb are differentially expressed in barley. Plant Physiology 118:37–49

Sun, C. Sathish, P. Ahlandsberg, S. and Jansson, C. (1999). Analyses of isoamylase gene activity in wild-type barley indicate its involvement in starch synthesis. Plant Molecular Biology 40: 431–443

Sun, C., Palmqvist, S., Olsson, H., Borén, M., Ahlandsberg, S and Jansson, C (2003). A novel WRKY transcription factor, SUSIBA2, participates in sugar signaling in barley by binding to the sugar-responsive elements of the *iso1* promoter. The Plant Cell, 15: 2076–2092

Swanston, J.S and Molina-Cano, J.L (2001). β-amylase activity and thermostability in two mutants derived from the malting barley cv. Triumph. Journal of Cereal Science, 33:155-161

Swanston, J.S., Ellis, R.P and Tiller, S.A. (1995). Effects of the waxy and increased amylose genes on total beta-glucan and extractable starch. Journal of Cereal Science 22: 265-273.

Switzer, R. C., Merril, C. R and Shifrin, S (1997). A highly sensitive silver stain for detecting proteins and peptides in polyacrylamide gels. Analytical Biochemistry. 98 (1) 231-237

Szczodrak, J and Pomeranz, Y (1991). Starch and enzyme-resistant starch from high-amylose barley. Cereal Chemistry 68:589–596.

Szydlowski, N., Ragel, P., Raynaud, S., Lucas, M. M., Roldan, I., Montero, M., Munoz, F. J., Ovecka, M., Bahaji, A., Planchot, V., Pozueta-Romero, J., D'Hulst, C and Merida, Á (2009). Starch granule initiation in Arabidopsis requires the presence of either Class IV or class III starch synthases. The Plant Cell 21: 2443–2457

Taira, T., Fujita, N., Takaoka, K., Uematsu, M., Wadano, A., Kozaki, S and Okabe, S. (1995). Variation in the primary structure of waxy proteins (Granule-Bound Starch Synthase) in diploid cereals. Biochemical Genetics, 33 (7/8): 269-281

Takaiwa, F., Yamanouchi, U., Yoshiara, T., Washida, H., Tanabe, F., Kato, A and Yamada,

K (1996). Characterization of common *cis*-regulatory elements responsible for the endosperm-specific expression of members of the rice glutelin multigene family. Plant Molecular Biology 30: 1207–1221.

Takeda Y, Shitaozono T and Hizukuri S. (1990). Structures of subfractions of corn amylose. Carbohydrate Research 199:207-214.

Takeda, Y., Guan, H. P and Preiss, J (1993) Branching of amylose by the branching isoenzymes of maize endosperm. Carbohydrate Research 240:253-263

Takahashi, R. (1964). Further studies on the phylogenetic differentiation of cultivated barley. Barley Genetics I: 19-26.

Tetlow, I. J., Davies, E. J., Vardy, K. A., Bowsher, C. G., Burrell, M. M and Michael Emes, J (2003). Subcellular localization of ADPglucose pyrophosphorylase in developing wheat endosperm and analysis of the properties of a plastidial isoform. Journal of Experimental Botany 54 (383): 715-725

Tetlow, I.J. , Morell, M.K and Emes, M. J (2004). Recent developments in understanding the regulation of starch metabolism in higher plants. J. Exp. Bot. 55:2131-2145.

Tetlow, l. J., Wait, R., Lu, Z., Akkasaeng, R., Bowsher, C. G., Esposito, S., Kosar-Hashemi, B., Morell, M. K and Emes, M. J (2004). Protein phosphorylation in amyloplasts regulates starch branching enzyme activity and protein-protein interactions. The Plant Cell, 16: 694-708

Tetlow, I. J., Beisel, K. G., Cameron, S., Makhmoudova, A., Liu, F., Bresolin, N. S., Wait, R., Morell, M. K. and Emes, M. J. (2008). Analysis of protein complexes in wheat amyloplasts reveals functional interactions among starch biosynthetic enzymes. Plant Physiology, 146: 1878–1891

Tang., H. Ando., H. Watanaba., K. Takeda., Y. Mitsunaga., T. (2001). Physicochemical properties and structure of large, medium and small granule starches in fractions of normal barley endosperm. Carbohydrate Research 330, 241–248.

Tang, H., Ando, H., Watanabe, K., Takeda, Y and Mitsunaga, T (1999). Some physicochemical properties of small-, medium- and large granules starches in fractions of waxy barley grain. Cereal Chemistry 77: 27-31.

Tang, M. C and Copeland, L. (2007). Investigation of starch retrogradation using atomic force microscopy. Carbohydrate Polymers 70[1]: 1-7.

Tanno, K and Takeda, K (2004). On the origin of six-rowed barley with brittle rachis, *agriocrithon* [*Hordeum vulgare ssp. vulgare f. agriocrithon* (Åberg) Bowd.], based on a DNA marker closely linked to the vrs1 (six-row gene) locus. Theoretical and Applied Genetics 110: 145–150.

Tester, R. F., Qi, X and Karkalas, J. (2006). Hydrolysis of native starches with amylases. Animal Feed Science and Technology, 130, 39-54.

Thomason, W. E., Brooks, W. S., Griffey, C. A., Vaughn, M. E., (2009). Hulless barley seeding rate effects on grain yield and yield components. Crop Science. 49, 342–346.

Thompson, J. D., Higgins, D.G and Gibson, T. J (1994). Clustal W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Research 22 (22): 4673-4680

Thompson, L. U and Yoon, J. H (1984). Starch digestibility as affected by polyphenol and phytic acid. Journal of Food Science 49:1228–9.

Thorbjørnsen T., Villand P., Denyer K., Olsen O-A and Smith A. M. (1996a). Distinct isoforms of ADPglucose pyrophosphorylase occur inside and outside the amyloplasts in barley endosperm. Plant Journal 10: 243–250

Tian, Y., Li, Y., Xu, X., Jin, Z., Jiao, A., Wang, J and Yu, B (2009). A novel size-exclusion high performance liquid chromatography (SE-HPLC) method for measuring degree of amylose retrogradation in rice starch. Food Chemistry. 111: 445-448

Tickle, P., Burrell, M. M., Coates, S. A., Emes, M. J., Tetlow, I. J and Bowsher, C. G (2009). Characterization of plastidial starch phosphorylase in *Triticum aestivum* L. endosperm. Journal of Plant Physiology 166: 1465-1478.

Topping, D. L and P. M. Clifton (2001). Short-chain fatty acids and human colonic function: roles of resistant starch and nonstarch polysaccharides. Physiology Review 81: 1031–1064.

Trowell. H. (1973). Dietary fiber: ischemic heart disease and diabetes mellitus. Proc. Nutr. Sot. 32: 151-155.

Tsai, C. J., Leitzmann, M. F., Willett, W. C and Giovannucci, E. L (2004). Long-term intake of dietary fiber and decreased risk of cholecystectomy in women. American Journal of Gastroenterol. 99(7):1364-70.

Tsai CY (1974). The function of the waxy locus in starch synthesis in maize endosperm. Biochemical Genetics, 11:83-96.

Tsuchiya, Y., Araki, S., Sahara, H., Takashio M. and Koshino, S (1995). Identification of malting barley varieties by genome analysis Journal of Fermentation and Bioengineering. 79 (5): 429-432

Turnbull, K. M and Rahman, S. (2002). Endosperm texture in wheat. Journal of Cereal Science 36, 327–337.

Ullrich, S. E (2002). Genetics and breeding of barley feed quality. In: Barley Science: Recent advances from molecular biology to agronomy of yield and quality. Slafer, G. A., Molina-Cano, J. L., Araus, J. L and Romagosa, I. (eds) Haworth Press, Binghamton, N.Y. pp 115-142.

Umemoto, T., Yano, M., Satoh, H., Shomura, A and Nakamura, Y (2002) Mapping of a gene responsible for the difference in amylopectin structure between japonioca-type and indica-type rice varieties. Theoretical and Applied Genetics 104: 1–8

Umemoto, T and Aoki, N (2005). Single nucleotide polymorphism in rice starch synthase IIa that alter starch gelatinization and starch association of the enzyme. Functional Plant Biology 32:763-768

US Department of Health and Human Services, Food and Drug Administration (2006). Food Lebelling: Health claims; soluble dietary fiber from certain foods and coronary heart disease. Federal Register Vol 71, N. 98: 29248-29250.

van de Wal, M., D'Huls,t C., Vincken, J-P., Buléon, A., Visser, R and Ball, S (1998). Amylose is synthesized in vitro by extension of and cleavage from amylopectin. Journal of Biological Chemistry 273:22232–40

Vasanthan, T. and Bhatty, R. S. (1995). Starch purification after pin milling and air classification of waxy, normal, and high amylase barleys. Cereal Chemistry 72: 379-384.

Vasanthan, T. and Bhatty, R. S. (1996). Physicochemical properties of small-and large- granule starches of waxy, regular and high amylase barleys. Cereal Chemistry 72: 199-207.

Vasanthan T and Temelli, F. (2008). Grain fractionation technologies for cereal beta-glucan concentration. Food Research International 41:876–881

Villand, P., Olsen, O. A., Kilian, A and Kleczkowski, L. A (1992). ADP-glucose

pyrophosphorylase large subunit cDNA from barley endosperm. Plant Physiol. 100(3): 1617–1618

Visser, R. G. F., Hergersberg, M., van der Leij, F. R., Jacobsen, E., Witholt, B and Feenstra, W. J (1989). Molecular cloning and partial characterisation of the gene for granule-bound starch synthase from a wild-type and an amylose-free potato (*Solanum tuberosum* L.). Plant Science 64:185-192.

Vrinten,, P. L and Nakamura, T (2000). Wheat granule-bound starch synthase l and ll are encoded by separate genes that are expressed in different tissues. Plant Physiology 122: 255–263.

Vorwerg, W., Radosta, S and Leibnitz, E (2002). Study of a preparative-scale process for the production of amylose. Carbohydrate Polymer 47:181-189.

Waduge, R, N., Hoover, R., Vasanthan, T., Gao, J and Li, J. (2006). Effect of annealing on the structure and physicochemical properties of barley starches with varying amylose content. Food Research international 39: 59-77.

Wang, Z., Wu, Z., Xing, Y., Zheng, F., Gao, J. and Hong, M (1990). Nucleotide sequence of rice waxy gene. Nucleic Acids Research 18: 5898.

Wang, Y. -J., White, P., Pollack, L and Jane, J. L (1993). Characterization of starch structures of 17 maize endosperm mutant genotypes with Oh43 inbred line background. Cereal Chemistry *70* (2): 171–179.

Wang, L., Behr, S. R., Rosemary, K., Newman, R. D and Newman, C. W (1997). Comparative cholesterol-lowering effects of barley β-glucan and barley oil in golden Syrian hamsters. Nutritional Research 17: 77–88.

Wang, J., Sporns, P and Low, N. H (1999). Analysis of food oligosaccharides using MALDI-MS: Quantification of fructo-oligosaccharides. Journal of Agriculture and Food Chemistry 47: 1549–1557.

Wang, L., Wang, Y. J and Porter, R (2002). Structures and physicochemical properties of six wild rice starches. Journal of Agriculture and Food Chemistry 50, 2695–2699.

Wang, J. M., Zhang, G. P., Chen, J. X., Shen, Q. Q and Wu, F. B (2003). Genotypic and environmental variation in barley beta-amylase activity and its relation to protein content. Food Chemistry 83:163–165.

Wang, Z., Chen, X., Wang, J., Liu, T., Liu, Y., Zhao, L and Wang, G (2007). Increasing maize seed weight by enhancing the cytoplasmic ADP-glucose pyrophosphorylase activity in transgenic maize plants. Plant Cell Tissue and Organ Culture 88:83–92.

Wang, S., Yu, J., Jin F and Yu, J (2008). The new insight on ultrastructure of C-type starch granules revealed by acid hydrolysis. Journal of Biological Macromolecules, 43 (2):216-220

Wang, C-P., Pan, Z-F., Nima, Z-X., Tang, Y-W., Cai, P., Liang, J-J., Deng, G-B., Long, H and Yu, M-Q (2010). Starch granule-associated proteins of hull-less barley (*Hordeum vulgare* L.) from the Qinghai-Tibet Plateau in China. Journal Science Food and Agriculture 91: 616–624.

Washington, J.M., A. Box, and A.R. Barr. (2000). Developing waxy barley cultivars for food, feed and malt. p. 303–306. *In* S. Logue (ed.) Barley genetics VIII. Int. Barley Genet. Symp. 8th, Adelaide, Australia. 22–27 Oct. 2000. Univ. of Adelaide, SA, Australia.

Wattebled, F., Buléon, A., Bouchet, B., Ral, J-P., Liénard, L., Delvallé, D., Binderup, K.,

Dauvillée, D., Ball, S. and D'Hulst, C. (2002). Granule-bound starch synthase I: A major enzyme involved in the biogenesis of B-crystallites in starch granules. European Journal of Biochemitry. 269, 3810–3820

Wei, C., Qin, F., Zhu, L., Zhou, W., Chen, Y., Wang, Y., Gu, M and Liu, Q (2010). Microstructure and ultrastructure of high-amylose rice resistant starch granules modified by antisense RNA inhibition of starch branching enzyme. Journal of Agriculture & Food Chemistry. 58:1224–1232.

Wei, C., Xu, B., Qin, F., Yu, H., Chen, C., Meng, X., Zhu, L., Wang, Y., M., Gu and Liu, Q (2010). C-Type Starch from High-Amylose Rice Resistant Starch Granules Modified by Antisense RNA Inhibition of Starch Branching Enzyme. Journal of Agriculture and Food Chemistry 58, 7383–7388

Weselake, R. J., Macgregor, A. W and Hill, R. D (1985). Effect of endogenous barley α-amylase inhibitor on hydrolysis of starch under various conditions. *J.Cereal Sci*., 3: 249-259.

Wessler, S.R and Varagona, M (1985). Molecular basis of mutations at the waxy locus of maize: Correlation with the fine structure genetic map. Proceedings of National Academy of Science USA 82:4177-4181

Wessler, S. R., Tarpley, A., Purugganan, M., Spell, M and Okagaki, R (1990). Filler DNA is associated with spontaneous deletion in maize. Proceedings of National Academy of Science USA 87:8731–8735

Wheelan, S. J., Church, D. M and Ostell, J. M (2001). Spidey: A Tool for mRNA-to-Genomic Alignments. Genome Research 11: 1952-1957

White, P.J. (2000). Dietary fiber. p. 201–213. *In* C.F. Murphy and D.M. Peterson (ed.) Designing crops for added value. Agron. Monogr. 40. ASA, CSSA, and SSSA, Madison, WI.

Winger, M., Christen, M and van Gunsteren, W. F (2009). On the conformational properties of amylose and cellulose oligomers in solution. International Journal of Carbohydrate Chemistry. doi:10.1155/2009/307695

Wood, P. J and Fulcher, R. G (1978). Interaction of some dyes with cereal β-glucans. Cereal Chemistry. 55, 952-966.

Wood, P. J., Beer, M. U and Butler, G (2000). Evaluation of role of concentration and molecular weight of oat beta-glucan in determining effect of viscosity on plasma glucose and insulin following an oral glucose load. British Journal of Nutrition. 84, 19–23.

Wood, P. J. (2002). Relationships between solution properties of cereal β-glucans and physiological effects—A review. Trends Food Science and Technology 13:313-320.

Wursch, P and Koellreutter, B (1992). Susceptibility of resistant starch to α- amylase. European Journal of Clinical Nutrition. 46: S113-S115.

Xue, Q., Wang, L., Newman, R. K., Newman, C. W., and Graham, H (1997). Influence of the hulles, waxy starch and short-awn genes on the composition of barleys. Journal of Cereal Science 26: 251-257.

Yadav B. S. . Sharma A and Yadav R. B (2010). Resistant starch content of conventionally boiled and pressure-cooked cereals, legumes and tubers. Journal of Food Science and Technology 47(1):84–88

Yamamori M, Fujita S, Hayakawa K, Matsuki J, Yasui T (2000). Genetic elimination of a starch granule protein, SGP-1, of wheat generates an altered starch with apparent increased amylose. Theoretical and Applied Genetics. 101:21-29

Yamamori, M., Nakamura, T., Endo, T. R., and Nagamine, T. (1994). Waxy protein deficiency in chromosomal location of coding genes in common wheat. Theoretical and Applied Genetics 89:179-184.

Yamamori, M., Nakamura, T., and Nagamine, T. (1995). Inheritance of waxy endosperm character in a common wheat lacking three Wx proteins. Breeding Sci. 45:377-379.

Yamanouchi, H. and Nakamura, Y. (1992). Organ specificity of isoforms of starch branching enzyme (Q-enzyme) in rice. Plant Cell Physiology 33: 985-991.

Yan, L., Bhave, M., Fairclough, R., Konik, C., Rahman, S and Appels, R. (2000). The genes encoding granule-bound starch synthases at the *waxy* loci of the A, B, and D progenitors of common wheat. Genome 43: 264–272.

Yanagisawa, T., Domon, E., Fujita, M., Kiribuchi-Otobe, C and Takayama, T (2006). Starch pasting properties and amylose content from 17 waxy barley lines. Cereal Chemistry 83(4):354–357.

Yano, M., Okuno, K., Kawakami, J., Satoh, H and Omura, T (1985). High amylose mutants of rice, *Oryza sativa* L. Theoretical and Applied Genetics 69, 253–257

Yao, N., Paez, A. V and White, P. J (2009). Structure and function of starch and resistant starch from corn with different doses of mutant amylose-extender and floury-1 alleles. *Journal of Agriculture and Food Chemistry* 57: 2040-2048.

Yep, A., Ballicora, M. A and Preiss, J (2004) The active site of the *Escherichia coli* glycogen synthase is similar to the active site of retaining GT-B glycosyltransferases. *Biochemical and Biophysical Research Communications* 316(3):960-966

Yin, C., Zhang, G.P., Wang, J.M and Chen, J.X., 2002. Variation of beta-amylase activity in barley as affected by cultivar and environment and its relation to protein content and grain weight. Journal of Cereal Science 36, 307–312.

Yoo, S. H and Jane, J (2002) Structural and physical characteristics of waxy and other wheat starches. Carbohydrate Polymers 49:297–305

You, S and Izydorczyk, M. S. (2002). Molecular characteristics of barley starches with variable amylose content. Carbohydrate Polymers 49: 33-42

Yun, S.H and Matheson, N.K (1993). Structure of the amylopectins of waxy, normal, amylose-extender, and wx:ae genotypes and of the phytoglycogen of maize. Carbohydrate Research 243 307-321.

Zeeman, S. C., Umemoto, T., Lue, W-L., Au-Yeung, P., Martin, C., Smith, A. M and Chen, (1998). A mutant of *Arabidopsis* lacking a chloroplastic isoamylase accumulates both starch and phytoglycogen. *Plant Cell* 10: 1699–1711.

Zhang G, Wang J, Chen J (2002) Analysis of β-glucan content in barley cultivars from different locations of China. Food Chemistry 79:251–254

Zhang, X., Colleoni, C., Ratushna, V., Sirghie-Colleoni, M., James, M.G., Myers, A.M (2004). Molecular characterization demonstrates that the Zea mays gene *sugary2* codes for the starch synthase isoform SSIIa. Plant Molecular Biology. 54 865-879

Zhang, G. P., Wang, J. M., Chen, J. X., Wu, F. B and Ding, S. R. (2005). The effect of cultivar and environment on beta-amylase activity is associated with the change of protein content in barley grains. Journal of Agronomy and Crop Science *192, 43-49*

Zhang, G.; Ao, Z and Hamaker, B. R (2006). Slow digestion property of native cereal starches. *Biomacromolecules 7*, 3252–3258.

Zhang, G., Venkatachalam, M and Hamaker, B. R (2006). Structural basis for the slow digestion property of native cereal starches. Biomacromolecules 7, 3259–3266.

Zhang, G, Ao Z, and Hamaker R. B. (2008). Nutritional property of endosperm starches from maize mutant: A parabolic relationship between slowly digestible starch and amylopectin fine structure. Journal of Agriculture and Food Chemistry 56: 4686-4694

Zhao X. C and Sharp PJ (1996). An improved 1-D SDS-PAGE method for the identification of three bread wheat 'waxy proteins. Journal of Cereal Science 23:191-193

Zheng, G. H and Sosulski, F. W. (1997). Physicochemical properties of small granule starches. AACC Annual Meeting, San Diego

Zheng, G. H and Sosulski, F. W (1998). Determination of water separation from cooked starch and flour pastes after refrigeration and freeze-thaw. Journal of Food Science 63, 134–139.

Zheng, G. H., Han, H. L and Bhatty, R. S (1998). Physiochemical properties of zero amylose hulless barley starch. *Cereal Chem*. 75: 520-524.

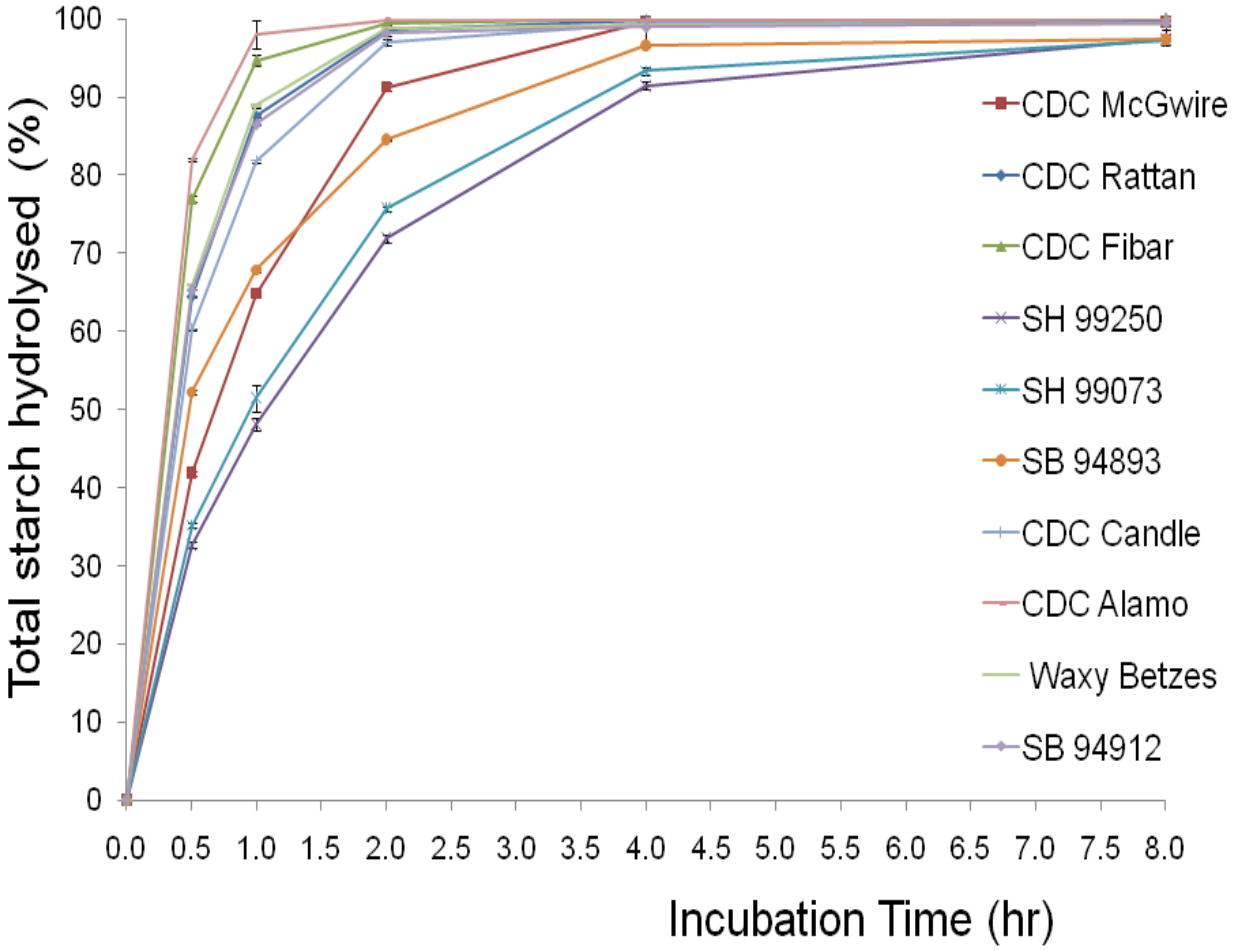
Zhong, F., Li, Y., Ibáňez, A. M., MoonHunOh, McKenzie, K. S and Shoemaker, C (2009). The effect of rice variety and starch isolation method on the pasting and rheological properties of rice starch pastes. Food Hydrocolloids 23:406–414

Zhong, F., Yokoyama, W., Wang, Q and Shoemaker, C. F (2006). Rice starch, amylopectin and amylose: Molecualr weight and solubility in dimethyl sulfoxide-based solvents. Journal of Food Chemistry. 54: 2320-2326.

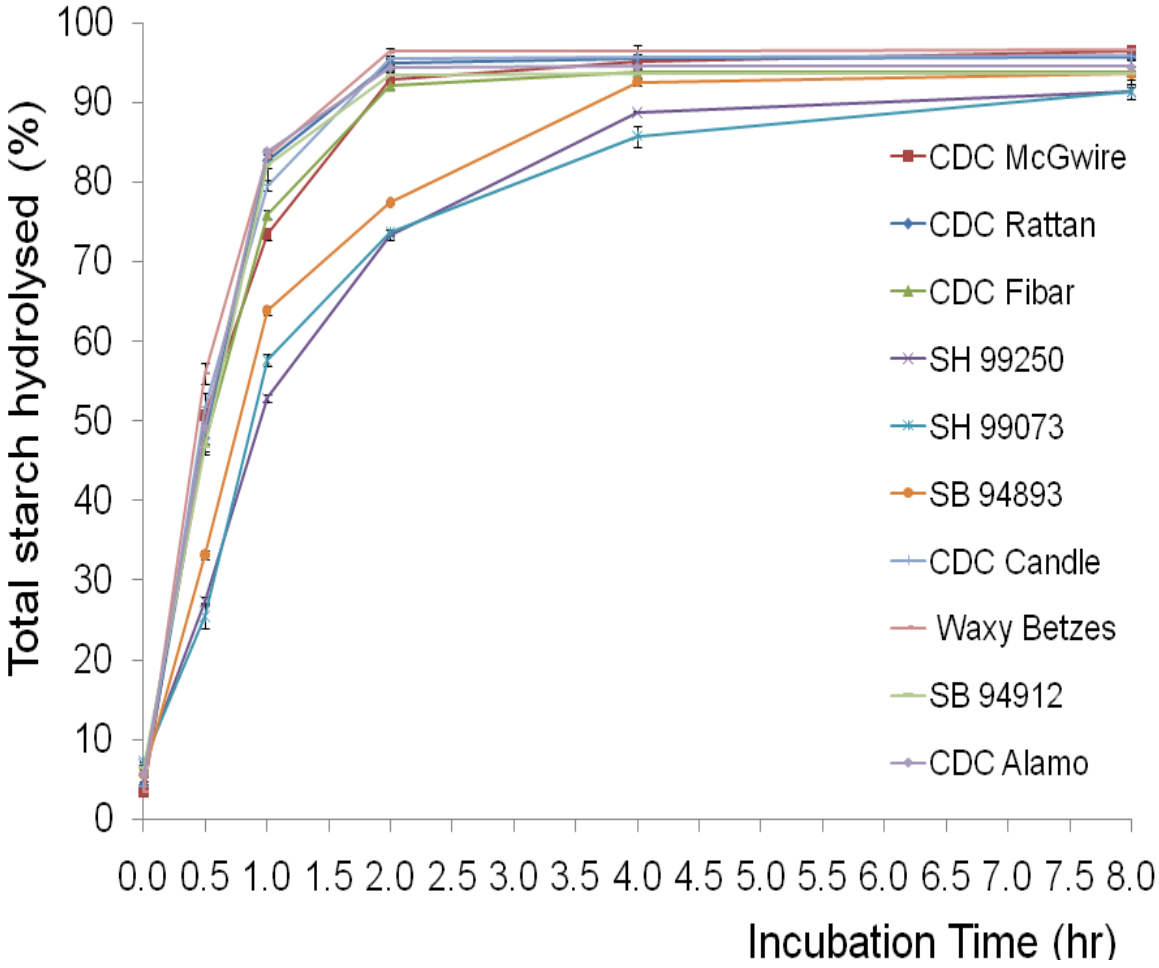
Zohary, D. (1964). Spontaneous brittle six-row barleys, their nature and origin. Barley Genetics I, Proc. 1st Int. Barley Genet. Symp., Wageningen, pp. 27-31

Zohary, D and Hopf, M (2000). Domestication of plants in the Old World, the origin and spread of cultivated plants in West Asia, Europe and the Vile Valley (3rd ed). Oxford University Press. Pp59-69

APPENDICES



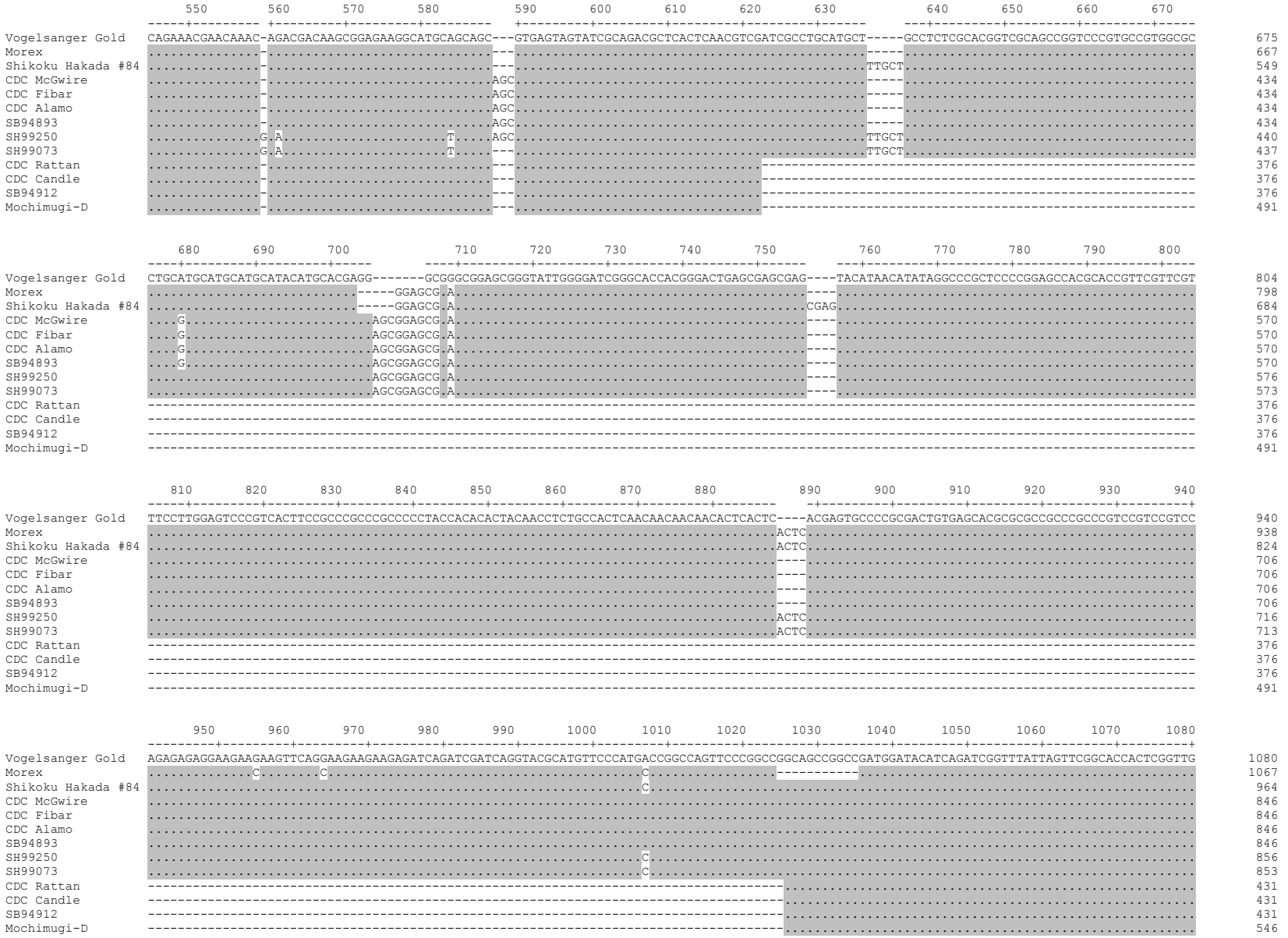
Appendix 3.1 *In-vitro* enzymatic hydrolysis curves of extracted starch.
The waxy (CDC Fibar and CDC Alamo) and near waxy (CDC Candle, CDC Rattan, Waxy Betzes and SB94912) genotypes showed rapid rate of hydrolysis within 4 hr (0 to 4 hr) compared to the normal (CDC McGwire) and increased amylose (SH99250, SH99073 and SB(94893). No significant differences in rate of hydrolysis were observed between genotypes from 4 hr to 8 hr.

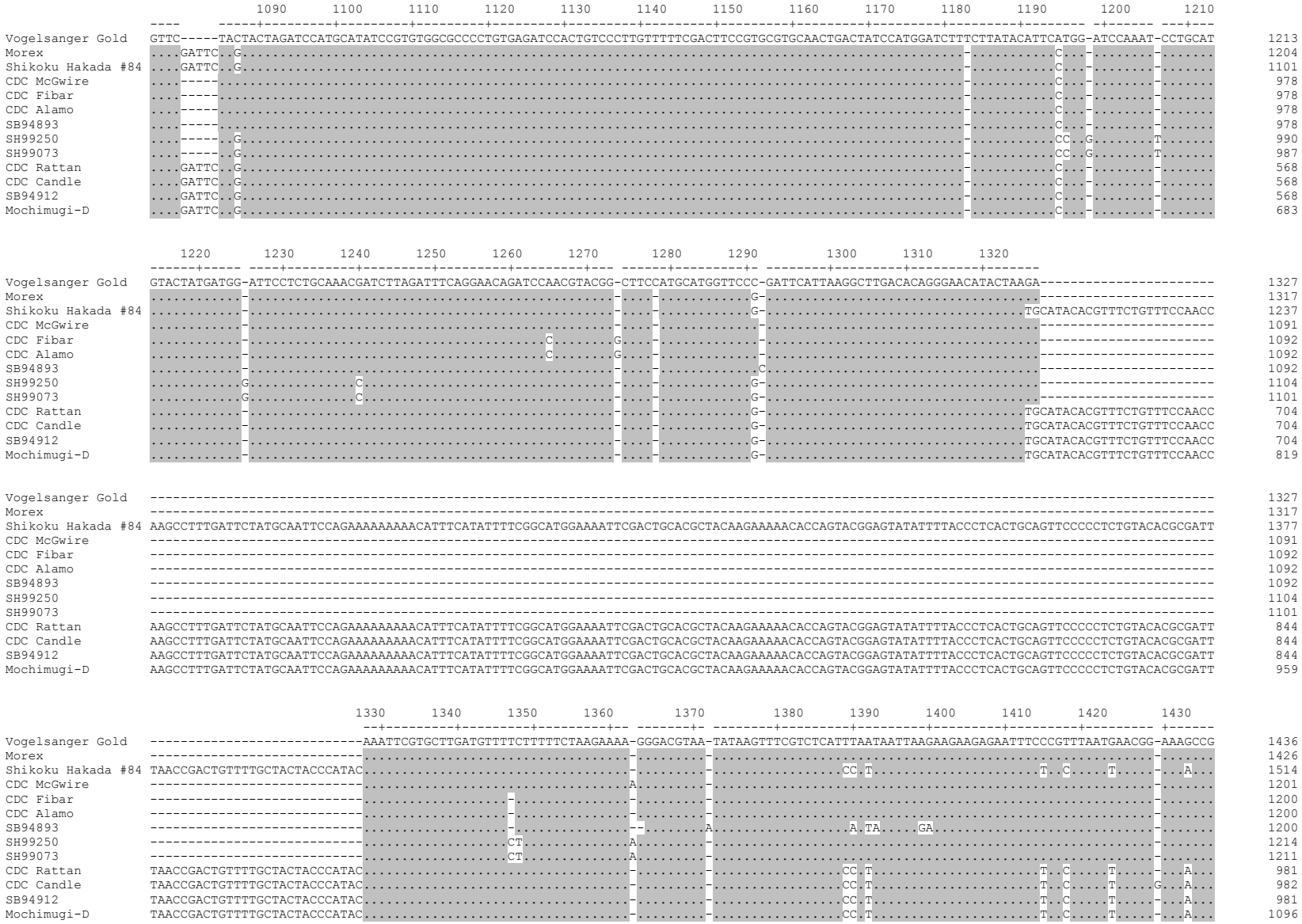


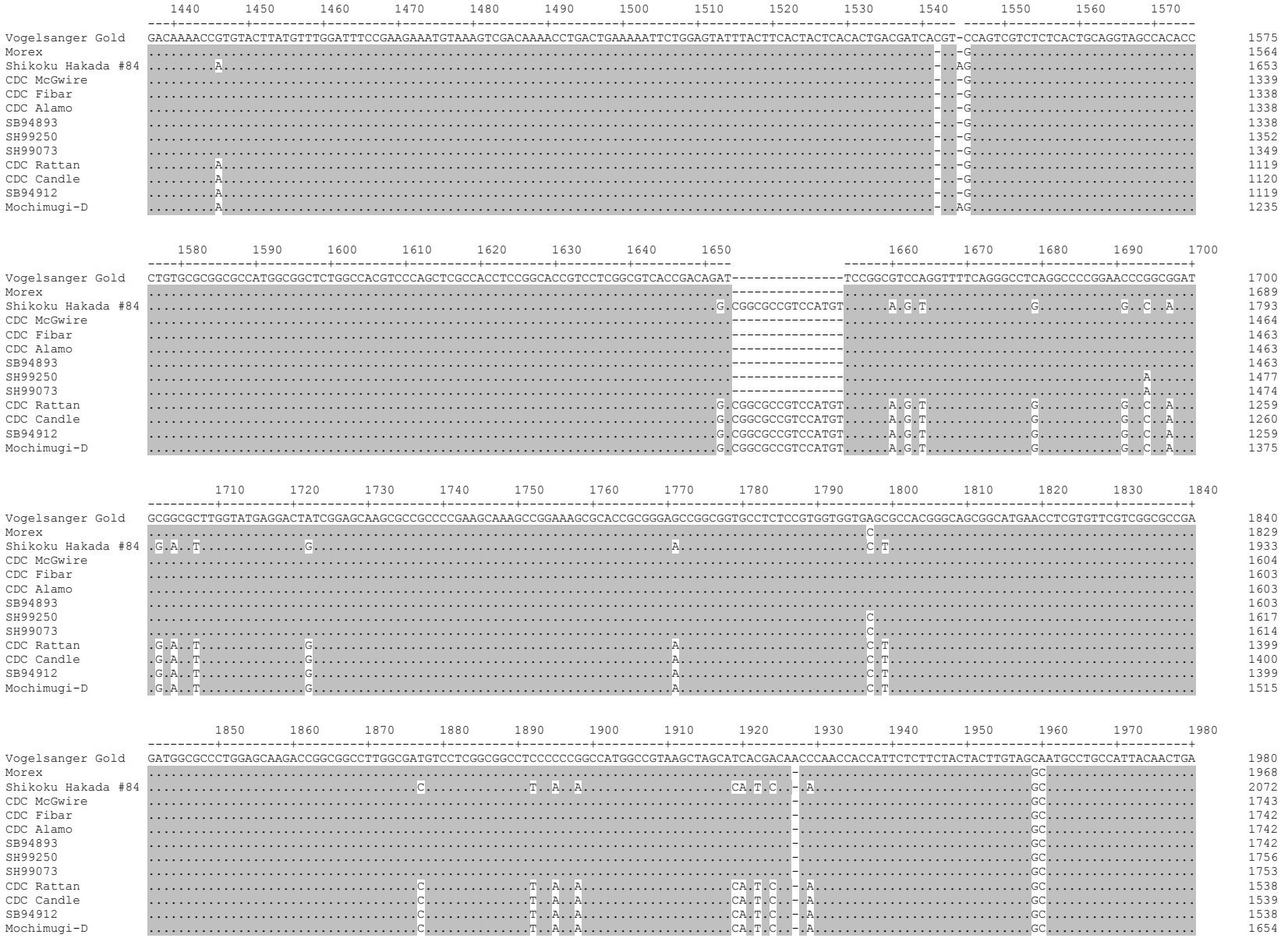
Appendix 3.2 *In-vitro* enzymatic hydrolysis curves of meal samples.
Hydrolysis of starch from the increased amylose (SH99250, SH99073 and SB(94893) genotypes were relatively slower from 0 to 4 hr of incubation compared to the waxy (CDC Fibar and CDC Alamo) and near waxy (CDC Candle, CDC Rattan, Waxy Betzes and SB94912) genotypes. The waxy and near waxy genotypes did not show any difference in hydrolysis rate from 2 hr to 8 hr.

4.1. DNA Sequence Alignemnt of PCR derived genomic DNA (genotypes in study) of *Gbss1* against other genotypes obtained from the GeneBank. Sequences were aligned using the clustalW method of the Megaalign program (DNASTAR). Identical nucleotides were indicated by (•) whereas nucleotides absent is denoted as (─). Nucleotide polymorphism is indicated in the respective base (s). Vogelsanger Gold (X07931, Rohde at al., 1988) a normal genotype used as a reference sequences to which all the other genotypes were compared. The other genotypes includes in the alignment were Morex (AF474373.1, SanMiguel et al., 2002), Shikoku Hakada #84 (AB088761, Patron et al., 2002) and Mochimogi-D (AB087716, Domon et al., 2002).

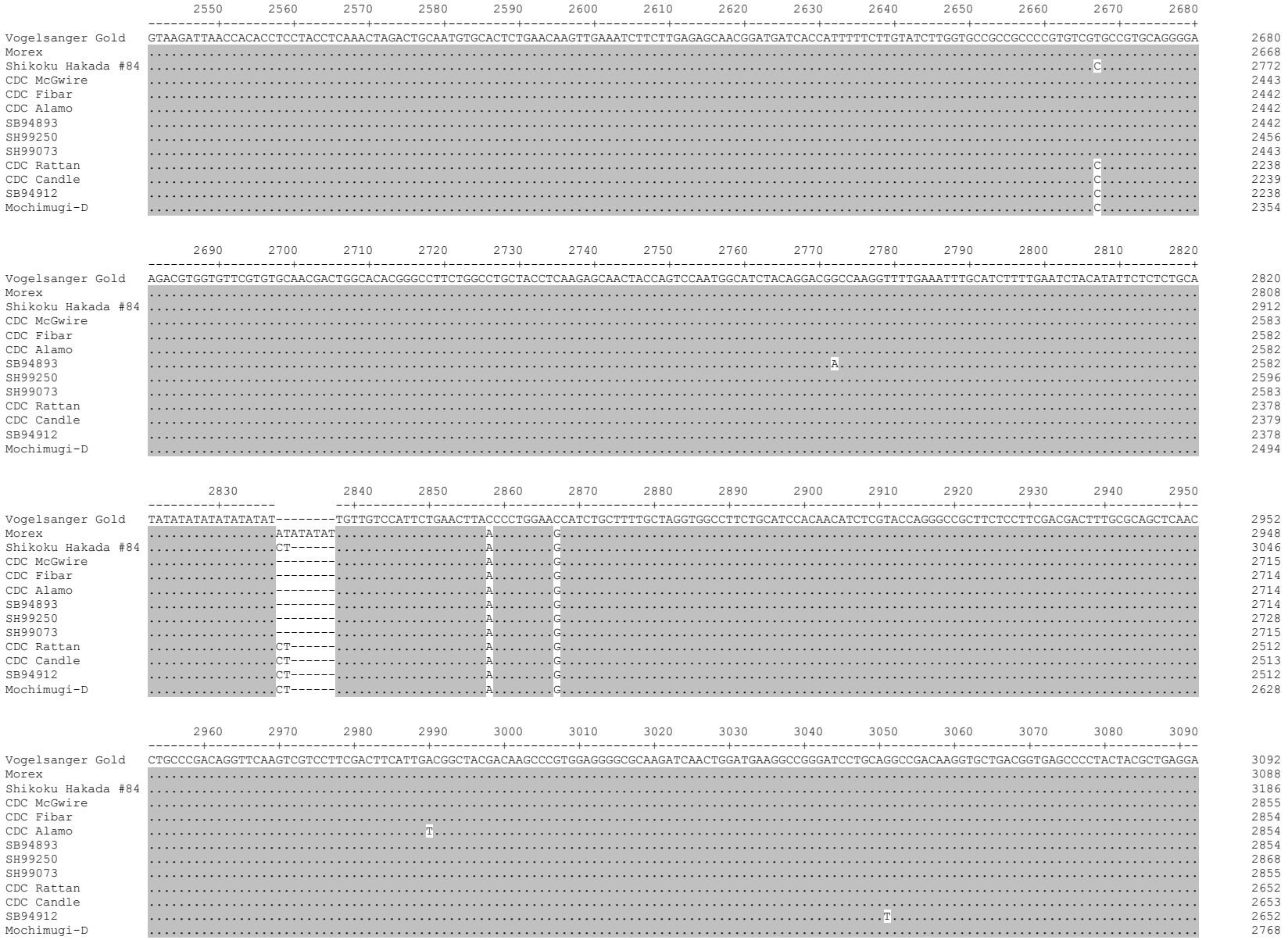

Vogelsanger Gold
Morex
Shikoku Hakada #84
CDC McGwire
CDC Fibar
CDC Alamo
SB94893
SH99250
SH99073
CDC Rattan
CDC Candle
SB94912
Mochimugi-D

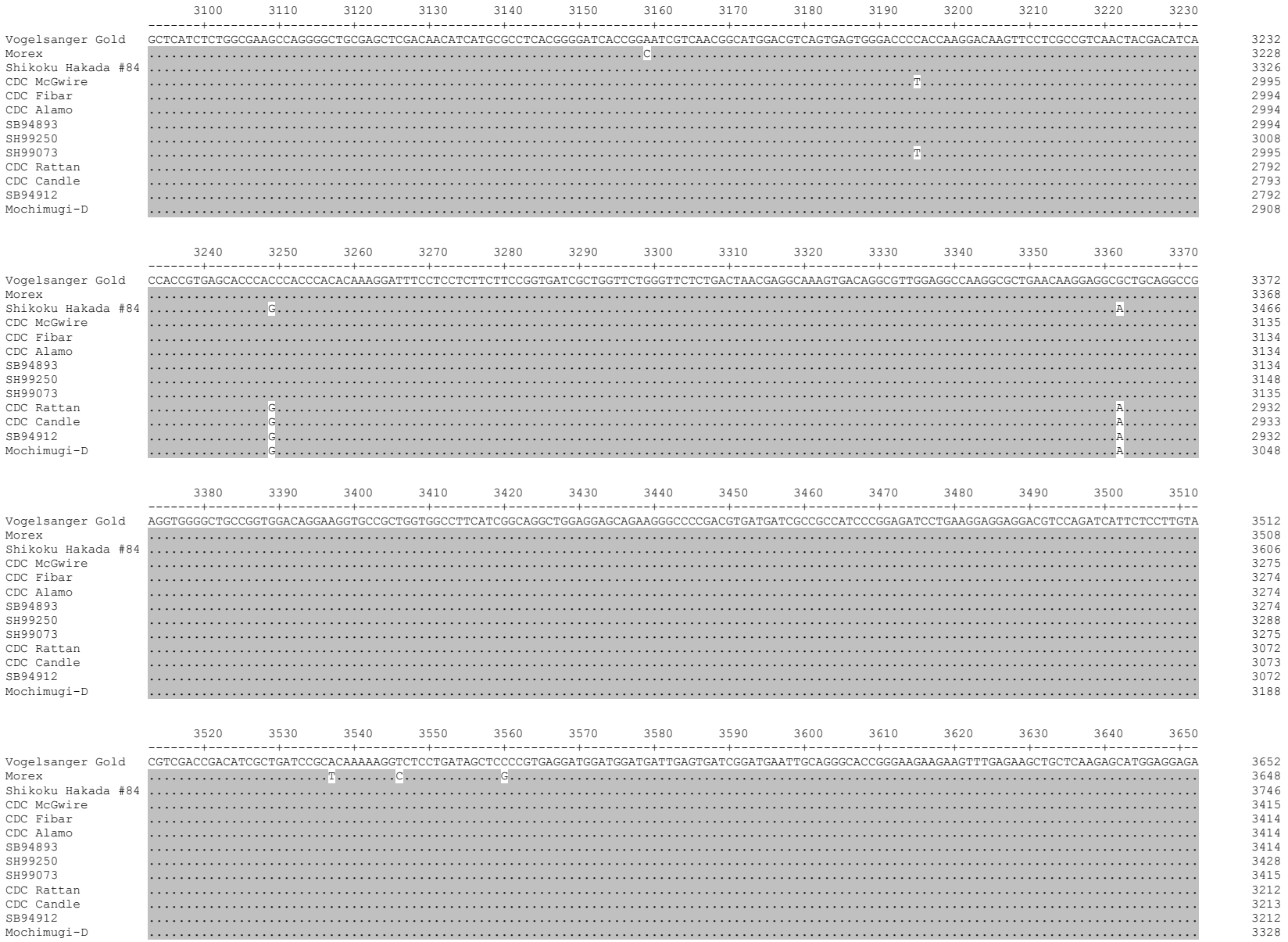

550 560 570 580 590 600 610 620 630 640 650 660 670
Vogelsanger Gold CAGAAACGAACAAAC-AGACGACAAGCGGAGAAGGCATGCAGCAGC---GTGAGTAGTATCGCAGACGCTCACTCAACGTCGATCGCCTGCATGCT-----GCCTCTCGCACGGTCGCAGCCGGTCCCGTGCCGTGGCGC 675
Morex 667
Shikoku Hakada #84 549
CDC McGwire 434
CDC Fibar 434
CDC Alamo 434
SB94893 434
SH99250 440
SH99073 437
CDC Rattan 376
CDC Candle 376
SB94912 376
Mochimugi-D 491
680 690 700 710 720 730 740 750 760 770 780 790 800
Vogelsanger Gold CTGCATGCATGCATGCATACATGCACGAGG-------GCGGGCGGAGCGGGTATTGGGGATCGGGCACCACGGGACTGAGCGAGCGAG----TACATAACATATAGGCCCGCTCCCCGGAGCCACGCACCGTTCGTTCGT 804
Morex 798
Shikoku Hakada #84 684
CDC McGwire 570
CDC Fibar 570
CDC Alamo 570
SB94893 570
SH99250 576
SH99073 573
CDC Rattan 376
CDC Candle 376
SB94912 376
Mochimugi-D 491
810 820 830 840 850 860 870 880 890 900 910 920 930 940
Vogelsanger Gold TTCCTTGGAGTCCCGTCACTTCCGCCCGCCCGCCCCCTACCACACACTACAACCTCTGCCACTCAACAACAACAACACTCACTC----ACGAGTGCCCCGCGACTGTGAGCACGCGCGCCGCCCGCCCGTCCGTCCGTCC 940
Morex 938
Shikoku Hakada #84 824
CDC McGwire 706
CDC Fibar 706
CDC Alamo 706
SB94893 706
SH99250 716
SH99073 713
CDC Rattan 376
CDC Candle 376
SB94912 376
Mochimugi-D 491
950 960 970 980 990 1000 1010 1020 1030 1040 1050 1060 1070 1080
Vogelsanger Gold AGAGAGAGGAAGAAGAAGTTCAGGAAGAAGAAGAGATCAGATCGATCAGGTACGCATGTTCCCATGACCGGCCAGTTCCCGGCCCGGCAGCCGGCCGATGGATACATCAGATCGGTTTATTAGTTCGGCACCACTCGGTTG 1080
Morex 1067
Shikoku Hakada #84 964
CDC McGwire 846
CDC Fibar 846
CDC Alamo 846
SB94893 846
SH99250 856
SH99073 853
CDC Rattan 431
CDC Candle 431
SB94912 431
Mochimugi-D 546

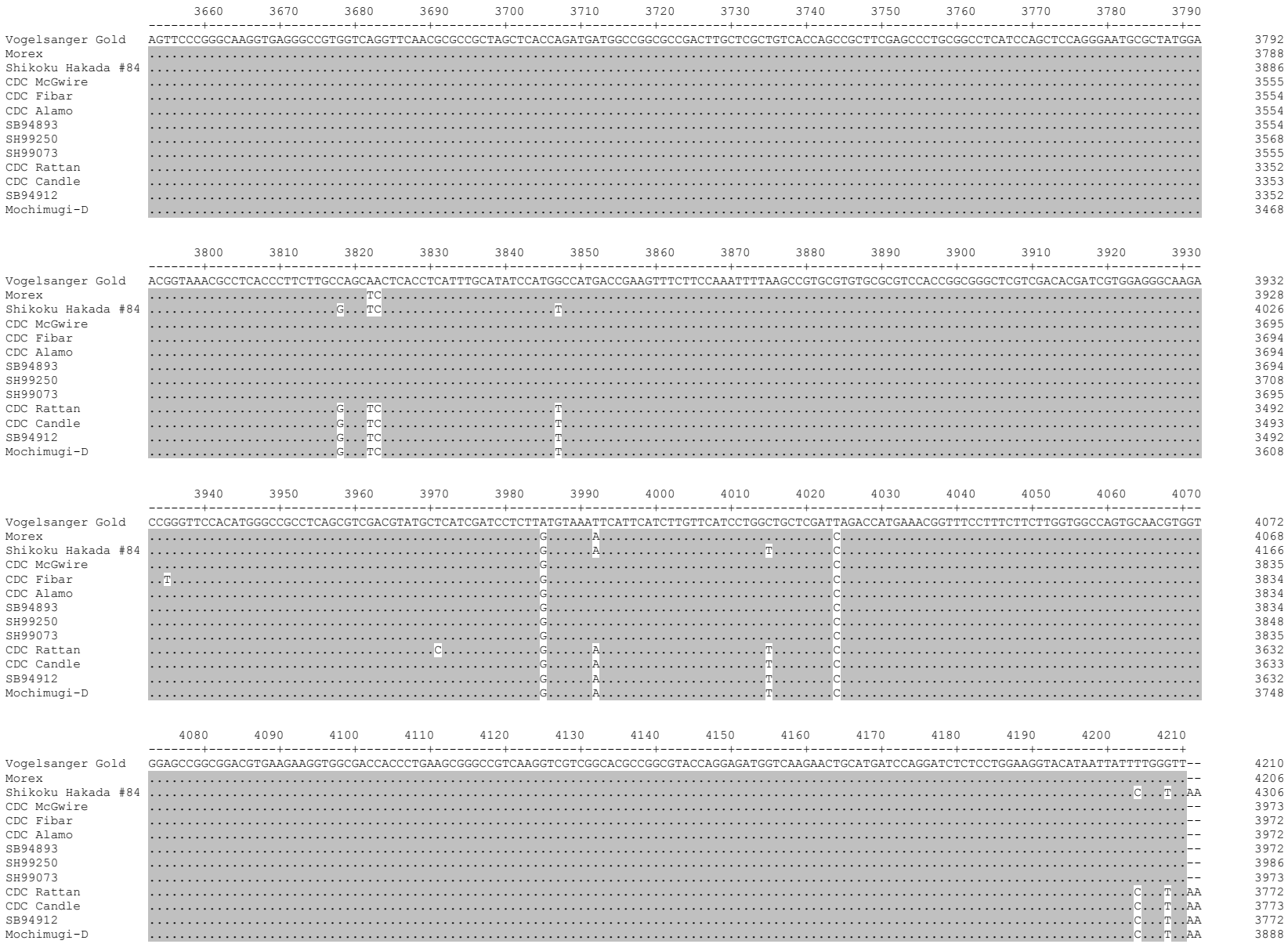

1090 1100 1110 1120 1130 1140 1150 1160 1170 1180 1190 1200 1210
Vogelsanger Gold GTTC-----TACTACTAGATCCATGCATATCCGTGTGGCGCCCCTGTGAGATCCACTGTCCCTTGTTTTTCGACTTCCGTGCGTGCAACTGACTATCCATGGATCTTTCTTATACATTCATGG-ATCCAAAT-CCTGCAT 1213
Morex ...GATTC..G 1204
Shikoku Hakada #84 ...GATTC..G 1101
CDC McGwire ...----- 978
CDC Fibar ...----- 978
CDC Alamo ...----- 978
SB94893 ...----- 978
SH99250 ...------..G 990
SH99073 ...------..G 987
CDC Rattan ...GATTC..G 568
CDC Candle ...GATTC..G 568
SB94912 ...GATTC..G 568
Mochimugi-D ...GATTC..G 683
1220 1230 1240 1250 1260 1270 1280 1290 1300 1310 1320
Vogelsanger Gold GTACTATGATGG-ATTCCTCTGCAAACGATCTTAGATTTCAGGAACAGATCCAACGTACGG-CTTCCATGCATGGTTCCC-GATTCATTAAGGCTTGACACAGGGAACATACTAAGA---------- 1327
Morex ----------- 1317
Shikoku Hakada #84 ----------- 1237
CDC McGwire ----------- 1091
CDC Fibar ----------- 1092
CDC Alamo ----------- 1092
SB94893 ----------- 1092
SH99250 ----------- 1104
SH99073 ----------- 1101
CDC Rattan ----------- 704
CDC Candle ----------- 704
SB94912 ----------- 704
Mochimugi-D ----------- 819
Vogelsanger Gold ---------- 1327
Morex ---------- 1317
Shikoku Hakada #84 AAGCCTTTGATTCTATGCAATTCCAGAAAAAAAAACATTTCATATTTTCGGCATGGAAAATTCGACTGCACGCTACAAGAAAAACACCAGTACGGAGTATATTTTACCCTCACTGCAGTTCCCCCTCTGTACACGCGATT 1377
CDC McGwire ---------- 1091
CDC Fibar ---------- 1092
CDC Alamo ---------- 1092
SB94893 ---------- 1092
SH99250 ---------- 1104
SH99073 ---------- 1101
CDC Rattan AAGCCTTTGATTCTATGCAATTCCAGAAAAAAAAACATTTCATATTTTCGGCATGGAAAATTCGACTGCACGCTACAAGAAAAACACCAGTACGGAGTATATTTTACCCTCACTGCAGTTCCCCCTCTGTACACGCGATT 844
CDC Candle AAGCCTTTGATTCTATGCAATTCCAGAAAAAAAAACATTTCATATTTTCGGCATGGAAAATTCGACTGCACGCTACAAGAAAAACACCAGTACGGAGTATATTTTACCCTCACTGCAGTTCCCCCTCTGTACACGCGATT 844
SB94912 AAGCCTTTGATTCTATGCAATTCCAGAAAAAAAAACATTTCATATTTTCGGCATGGAAAATTCGACTGCACGCTACAAGAAAAACACCAGTACGGAGTATATTTTACCCTCACTGCAGTTCCCCCTCTGTACACGCGATT 844
Mochimugi-D AAGCCTTTGATTCTATGCAATTCCAGAAAAAAAAACATTTCATATTTTCGGCATGGAAAATTCGACTGCACGCTACAAGAAAAACACCAGTACGGAGTATATTTTACCCTCACTGCAGTTCCCCCTCTGTACACGCGATT 959
1330 1340 1350 1360 1370 1380 1390 1400 1410 1420 1430
Vogelsanger Gold ----------AAATTCGTGCTTGATGTTTTCTTTTTCTAAGAAAA-GGGACGTAA-TATAAGTTTCGTCTCATTTAATAATTAAGAAGAAGAGAATTTCCCGTTTAATGAACGG-AAAGCCG 1436
Morex ---------- 1426
Shikoku Hakada #84 TAACCGACTGTTTTGCTACTACCCATAC 1514
CDC McGwire ---------- 1201
CDC Fibar ---------- 1200
CDC Alamo ---------- 1200
SB94893 ---------- 1200
SH99250 ---------- 1214
SH99073 ---------- 1211
CDC Rattan TAACCGACTGTTTTGCTACTACCCATAC 981
CDC Candle TAACCGACTGTTTTGCTACTACCCATAC 982
SB94912 TAACCGACTGTTTTGCTACTACCCATAC 981
Mochimugi-D TAACCGACTGTTTTGCTACTACCCATAC 1096


1440 1450 1460 1470 1480 1490 1500 1510 1520 1530 1540 1550 1560 1570
Vogelsanger Gold GACAAAACCGTGTACTTATGTTTGGATTTCCGAAGAAATGTAAAGTCGACAAAACCTGACTGAAAAATTCTGGAGTATTTACTTCACTACTCACACTGACGATCACGT-CCAGTCGTCTCTCACTGCAGGTAGCCACACC 1575
Morex 1564
Shikoku Hakada #84 1653
CDC McGwire 1339
CDC Fibar 1338
CDC Alamo 1338
SB94893 1338
SH99250 1352
SH99073 1349
CDC Rattan 1119
CDC Candle 1120
SB94912 1119
Mochimugi-D 1235
1580 1590 1600 1610 1620 1630 1640 1650 1660 1670 1680 1690 1700
Vogelsanger Gold CTGTGCGCGGCGCCATGGCGGCTCTGGCCACGTCCCAGCTCGCCACCTCCGGCACCGTCCTCGGCGTCACCGACAGAT----------TCCGGCGTCCAGGTTTTCAGGGCCTCAGGCCCCGGAACCCGGCGGAT 1700
Morex 1689
Shikoku Hakada #84 1793
CDC McGwire 1464
CDC Fibar 1463
CDC Alamo 1463
SB94893 1463
SH99250 1477
SH99073 1474
CDC Rattan 1259
CDC Candle 1260
SB94912 1259
Mochimugi-D 1375
1710 1720 1730 1740 1750 1760 1770 1780 1790 1800 1810 1820 1830 1840
Vogelsanger Gold GCGGCGCTTGGTATGAGGACTATCGGAGCAAGCGCCGCCCCGAAGCAAAGCCGGAAAGCGCACCGCGGGAGCCGGCGGTGCCTCTCCGTGGTGGTGAGCGCCACGGGCAGCGGCATGAACCTCGTGTTCGTCGGCGCCGA 1840
Morex 1829
Shikoku Hakada #84 1933
CDC McGwire 1604
CDC Fibar 1603
CDC Alamo 1603
SB94893 1603
SH99250 1617
SH99073 1614
CDC Rattan 1399
CDC Candle 1400
SB94912 1399
Mochimugi-D 1515
1850 1860 1870 1880 1890 1900 1910 1920 1930 1940 1950 1960 1970 1980
Vogelsanger Gold GATGGCGCCCTGGAGCAAGACCGGCGGCCTTGGCGATGTCCTCGGCGGCCTCCCCCCGGCCATGGCCGTAAGCTAGCATCACGACAACCCAACCACCATTCTCTTCTACTACTTGTAGCAATGCCTGCCATTACAACTGA 1980
Morex 1968
Shikoku Hakada #84 2072
CDC McGwire 1743
CDC Fibar 1742
CDC Alamo 1742
SB94893 1742
SH99250 1756
SH99073 1753
CDC Rattan 1538
CDC Candle 1539
SB94912 1538
Mochimugi-D 1654

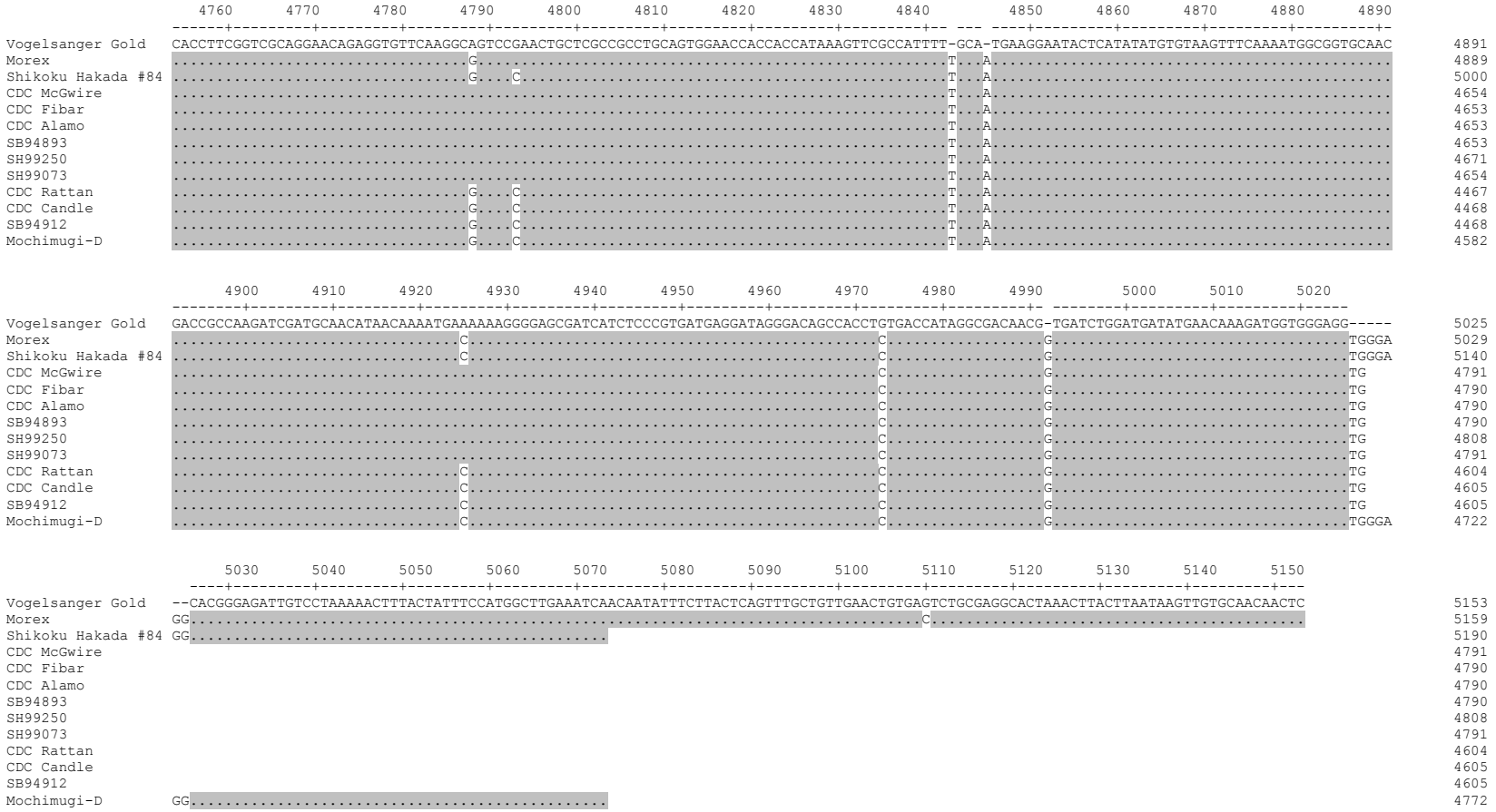

1990 2000 2010 2020 2030 2040 2050 2060 2070 2080 2090 2100 2110 2120
Vogelsanger Gold CGGCGTGTCTGTTCAGGCCAACGGTCACCGGGTGATGGTCGTCTCCCCGCGCTACGATCAGTACAAGGACGCCTGGGACACCAGCGTCATCTCCGAGGTAGGTATCCGCCATATGAATGATCACGCTTCACATGCTCCTG 2120
Morex 2108
Shikoku Hakada #84 T 2212
CDC McGwire 1883
CDC Fibar 1882
CDC Alamo 1882
SB94893 1882
SH99250 1896
SH99073 1893
CDC Rattan T 1678
CDC Candle T 1679
SB94912 T 1678
Mochimugi-D T 1794
2130 2140 2150 2160 2170 2180 2190 2200 2210 2220 2230 2240 2250 2260
Vogelsanger Gold CACATTTCTGCAAGATTCTACCGACTGGCTGGATTTCGCAGATCAAGGTCGCTGACGAGTACGAGAGGGTGAGGTTCTTCCACTGCTACAAGCGCGGAGTGGACCGCGTGTTCATCGACCACCCGTGGTTCCTGGAGAAG 2260
Morex A 2248
Shikoku Hakada #84 G 2352
CDC McGwire 2023
CDC Fibar 2022
CDC Alamo 2022
SB94893 2022
SH99250 2036
SH99073 2033
CDC Rattan G 1818
CDC Candle G 1819
SB94912 G 1818
Mochimugi-D G 1934
2270 2280 2290 2300 2310 2320 2330 2340 2350 2360 2370 2380 2390 2400
Vogelsanger Gold GTGCGTGACCGATTCTCGTCGTCGATCGATCGATCAAGCTATCTCTTTGTCCTACAAATTCCAAACTCAAAGGTGCATGGTGATTGATGTCAGTGAATCTTTGTTTCTGCTGCTTACAATTTCTAGGTCCGGGGCAAGAC 2400
Morex C 2388
Shikoku Hakada #84 G C 2492
CDC McGwire C 2163
CDC Fibar C 2162
CDC Alamo C 2162
SB94893 C 2162
SH99250 CT TC A AA TCC T CA T CC C 2176
SH99073 ------ CT TC A AA TCC T CA ---- T CC C 2163
CDC Rattan G C 1958
CDC Candle G C 1959
SB94912 G C 1958
Mochimugi-D G C 2074
2410 2420 2430 2440 2450 2460 2470 2480 2490 2500 2510 2520 2530 2540
Vogelsanger Gold CAAGGAGAAGATCTACGGGCCCGACGCCGGCACAGACTATGAGGACAACCAGCAGCGCTTCAGCCTTCTCTGCCAGGCAGCACTCGAGGCACCCAGGATCCTCAACCTCAACAACAACCCCTACTTTTCTGGTCCCTACG 2540
Morex 2528
Shikoku Hakada #84 G C G 2632
CDC McGwire 2303
CDC Fibar 2302
CDC Alamo 2302
SB94893 2302
SH99250 2316
SH99073 2303
CDC Rattan G C G 2098
CDC Candle G C G 2099
SB94912 G C G 2098
Mochimugi-D G C G 2214


2550 2560 2570 2580 2590 2600 2610 2620 2630 2640 2650 2660 2670 2680
Vogelsanger Gold GTAAGATTAACCACACCTCCTACCTCAAACTAGACTGCAATGTGCACTCTGAACAAGTTGAAATCTTCTTGAGAGCAACGGATGATCACCATTTTTCTTGTATCTTGGTGCCGCCGCCCCGTGTCGTGCCGTGCAGGGGA 2680
Morex 2668
Shikoku Hakada #84 2772
CDC McGwire 2443
CDC Fibar 2442
CDC Alamo 2442
SB94893 2442
SH99250 2456
SH99073 2443
CDC Rattan 2238
CDC Candle 2239
SB94912 2238
Mochimugi-D 2354
2690 2700 2710 2720 2730 2740 2750 2760 2770 2780 2790 2800 2810 2820
Vogelsanger Gold AGACGTGGTGTTCGTGTGCAACGACTGGCACACGGGCCTTCTGGCCTGCTACCTCAAGAGCAACTACCAGTCCAATGGCATCTACAGGACGGCCAAGGTTTTGAAATTTGCATCTTTTGAATCTACATATTCTCTCTGCA 2820
Morex 2808
Shikoku Hakada #84 2912
CDC McGwire 2583
CDC Fibar 2582
CDC Alamo 2582
SB94893 2582
SH99250 2596
SH99073 2583
CDC Rattan 2378
CDC Candle 2379
SB94912 2378
Mochimugi-D 2494
2830 2840 2850 2860 2870 2880 2890 2900 2910 2920 2930 2940 2950
Vogelsanger Gold TATATATATATATATAT--------TGTTGTCCATTCTGAACTTACCCCTGGAACCATCTGCTTTTGCTAGGTGGCCTTCTGCATCCACAACATCTCGTACCAGGGCCGCTTCTCCTTCGACGACTTTGCGCAGCTCAAC 2952
Morex 2948
Shikoku Hakada #84 3046
CDC McGwire 2715
CDC Fibar 2714
CDC Alamo 2714
SB94893 2714
SH99250 2728
SH99073 2715
CDC Rattan 2512
CDC Candle 2513
SB94912 2512
Mochimugi-D 2628
2960 2970 2980 2990 3000 3010 3020 3030 3040 3050 3060 3070 3080 3090
Vogelsanger Gold CTGCCCGACAGGTTCAAGTCGTCCTTCGACTTCATTGACGGCTACGACAAGCCCGTGGAGGGGCGCAAGATCAACTGGATGAAGGCCGGGATCCTGCAGGCCGACAAGGTGCTGACGGTGAGCCCCTACTACGCTGAGGA 3092
Morex 3088
Shikoku Hakada #84 3186
CDC McGwire 2855
CDC Fibar 2854
CDC Alamo 2854
SB94893 2854
SH99250 2868
SH99073 2855
CDC Rattan 2652
CDC Candle 2653
SB94912 2652
Mochimugi-D 2768


3100 3110 3120 3130 3140 3150 3160 3170 3180 3190 3200 3210 3220 3230
Vogelsanger Gold GCTCATCTCTGGCGAAGCCAGGGGCTGCGAGCTCGACAACATCATGCGCCTCACGGGGATCACCGGAATCGTCAACGGCATGGACGTCAGTGAGTGGGACCCCACCAAGGACAAGTTCCTCGCCGTCAACTACGACATCA 3232
Morex C 3228
Shikoku Hakada #84 3326
CDC McGwire T 2995
CDC Fibar 2994
CDC Alamo 2994
SB94893 2994
SH99250 3008
SH99073 T 2995
CDC Rattan 2792
CDC Candle 2793
SB94912 2792
Mochimugi-D 2908
3240 3250 3260 3270 3280 3290 3300 3310 3320 3330 3340 3350 3360 3370
Vogelsanger Gold CCACCGTGAGCACCCACCCACCCACACAAAGGATTTCCTCCTCTTCTTCCGGTGATCGCTGGTTCTGGGTTCTCTGACTAACGAGGCAAAGTGACAGGCGTTGGAGGCCAAGGCGCTGAACAAGGAGGCGCTGCAGGCCG 3372
Morex 3368
Shikoku Hakada #84 G A 3466
CDC McGwire 3135
CDC Fibar 3134
CDC Alamo 3134
SB94893 3134
SH99250 3148
SH99073 3135
CDC Rattan G A 2932
CDC Candle G A 2933
SB94912 G A 2932
Mochimugi-D G A 3048
3380 3390 3400 3410 3420 3430 3440 3450 3460 3470 3480 3490 3500 3510
Vogelsanger Gold AGGTGGGGCTGCCGGTGGACAGGAAGGTGCCGCTGGTGGCCTTCATCGGCAGGCTGGAGGAGCAGAAGGGCCCCGACGTGATGATCGCCGCCATCCCGGAGATCCTGAAGGAGGAGGACGTCCAGATCATTCTCCTTGTA 3512
Morex 3508
Shikoku Hakada #84 3606
CDC McGwire 3275
CDC Fibar 3274
CDC Alamo 3274
SB94893 3274
SH99250 3288
SH99073 3275
CDC Rattan 3072
CDC Candle 3073
SB94912 3072
Mochimugi-D 3188
3520 3530 3540 3550 3560 3570 3580 3590 3600 3610 3620 3630 3640 3650
Vogelsanger Gold CGTCGACCGACATCGCTGATCCGCACAAAAAGGTCTCCTGATAGCTCCCCGTGAGGATGGATGGATGATTGAGTGATCGGATGAATTGCAGGGCACCGGGAAGAAGAAGTTTGAGAAGCTGCTCAAGAGCATGGAGGAGA 3652
Morex T C G 3648
Shikoku Hakada #84 3746
CDC McGwire 3415
CDC Fibar 3414
CDC Alamo 3414
SB94893 3414
SH99250 3428
SH99073 3415
CDC Rattan 3212
CDC Candle 3213
SB94912 3212
Mochimugi-D 3328


3660 3670 3680 3690 3700 3710 3720 3730 3740 3750 3760 3770 3780 3790
Vogelsanger Gold AGTTCCCGGGCAAGGTGAGGGCCGTGGTCAGGTTCAACGCGCCGCTAGCTCACCAGATGATGGCCGGCGCCGACTTGCTCGCTGTCACCAGCCGCTTCGAGCCCTGCGGCCTCATCCAGCTCCAGGGAATGCGCTATGGA 3792
Morex 3788
Shikoku Hakada #84 3886
CDC McGwire 3555
CDC Fibar 3554
CDC Alamo 3554
SB94893 3554
SH99250 3568
SH99073 3555
CDC Rattan 3352
CDC Candle 3353
SB94912 3352
Mochimugi-D 3468
3800 3810 3820 3830 3840 3850 3860 3870 3880 3890 3900 3910 3920 3930
Vogelsanger Gold ACGGTAAACGCCTCACCCTTCTTGCCAGCAACTCACCTCATTTGCATATCCATGGCCATGACCGAAGTTTCTTCCAAATTTTAAGCCGTGCGTGTGCGCGTCCACCGGCGGGCTCGTCGACACGATCGTGGAGGGCAAGA 3932
Morex 3928
Shikoku Hakada #84 4026
CDC McGwire 3695
CDC Fibar 3694
CDC Alamo 3694
SB94893 3694
SH99250 3708
SH99073 3695
CDC Rattan 3492
CDC Candle 3493
SB94912 3492
Mochimugi-D 3608
3940 3950 3960 3970 3980 3990 4000 4010 4020 4030 4040 4050 4060 4070
Vogelsanger Gold CCGGGTTCCACATGGGCCGCCTCAGCGTCGACGTATGCTCATCGATCCTCTTATGTAAATTCATTCATCTTGTTCATCCTGGCTGCTCGATTAGACCATGAAACGGTTTCCTTTCTTCTTGGTGGCCAGTGCAACGTGGT 4072
Morex 4068
Shikoku Hakada #84 4166
CDC McGwire 3835
CDC Fibar 3834
CDC Alamo 3834
SB94893 3834
SH99250 3848
SH99073 3835
CDC Rattan 3632
CDC Candle 3633
SB94912 3632
Mochimugi-D 3748
4080 4090 4100 4110 4120 4130 4140 4150 4160 4170 4180 4190 4200 4210
Vogelsanger Gold GGAGCCGGCGGACGTGAAGAAGGTGGCGACCACCCTGAAGCGGGCCGTCAAGGTCGTCGGCACGCCGGCGTACCAGGAGATGGTCAAGAACTGCATGATCCAGGATCTCTCCTGGAAGGTACATAATTATTTTGGGTT-- 4210
Morex -- 4206
Shikoku Hakada #84 AA 4306
CDC McGwire -- 3973
CDC Fibar -- 3972
CDC Alamo -- 3972
SB94893 -- 3972
SH99250 -- 3986
SH99073 -- 3973
CDC Rattan AA 3772
CDC Candle AA 3773
SB94912 AA 3772
Mochimugi-D AA 3888


4220 4230 4240 4250 4260 4270 4280 4290 4300 4310 4320 4330 4340
Vogelsanger Gold -------TCACGCAGCAATTTAAAGACTGCATGGCTCAATGGTGGTCTGGGCGTATGCTGCAGGGACCTGCCAAGAACTGGGAGGACGTGCTTCTGGAACTGGGGGTGGAGGGGAGCGAGCCGGGGATCGTCGGCGAGGA 4343
Morex ------- 4339
Shikoku Hakada #84 TGGTGAT.TT.A 4446
CDC McGwire ------- 4106
CDC Fibar ------- 4105
CDC Alamo ------- 4105
SB94893 ------- 4105
SH99250 ------- 4119
SH99073 ------- 4106
CDC Rattan TGGTGAT.TT.A 3912
CDC Candle TGGTGAT.TT.A 3913
SB94912 TGGTGAT.TT.A 3912
Mochimugi-D TGGTGAT.TT.A 4028
4350 4360 4370 4380 4390 4400 4410 4420 4430 4440 4450 4460 4470 4480
Vogelsanger Gold GATCGCGCCGCTCGCCATGGAGAACGTCGCCGCTCCCTGAAGAGAGA--GAAAAAGGAACATTCTGGTGCATGGAGCATCTTCCATCTTCAGGGTTCTCGTATGGGGAGATAGCCGCTTGTTGTAGTGAAGGGCCGATAT 4481
Morex AA. 4479
Shikoku Hakada #84 AA. 4586
CDC McGwire -- 4244
CDC Fibar -- 4243
CDC Alamo -- 4243
SB94893 -- 4243
SH99250 -- 4257
SH99073 -- 4244
CDC Rattan AA. 4052
CDC Candle AA. 4053
SB94912 AA. 4052
Mochimugi-D AA. 4168
4490 4500 4510 4520 4530 4540 4550 4560 4570 4580 4590 4600 4610
Vogelsanger Gold ATATAGACTAATAAGTACTTAAGTTTTGTTGTGCCGCTTGCCTCTTTACAAACCAAAAAAAAAAA------GTTAGGGGTTGTGCTTGTGACAAAGTGTGTATTTCATTTTGCTGTGGGTATATTGCAAATAAGCCAAGG 4615
Morex A----- 4614
Shikoku Hakada #84 AAAA-- 4724
CDC McGwire A----- 4379
CDC Fibar A----- 4378
CDC Alamo A----- 4378
SB94893 A----- 4378
SH99250 A----- 4392
SH99073 A----- 4379
CDC Rattan AAAAA- 4191
CDC Candle AAAAA- 4192
SB94912 AAAAAA 4192
Mochimugi-D AAAA-- 4306
4620 4630 4640 4650 4660 4670 4680 4690 4700 4710 4720 4730 4740 4750
Vogelsanger Gold ATTTGTTATGTGTTTTTGCTATTGTTTCAAAGTCTTTGTGTTTGATTCCATTTTGAGTTATTTTGTGGG-TTCCAAAGGAAAGGATCAGGGTTCATCGGGACATGACCTTGCATTTTTTT-GTGTTCATCATCGAGGTGG 4753
Morex ----G-T- 4749
Shikoku Hakada #84 ----G-T- 4860
CDC McGwire ----G-T- 4514
CDC Fibar ----G-T- 4513
CDC Alamo ----G-T- 4513
SB94893 ----G-T- 4513
SH99250 ----G-T- 4531
SH99073 ----G-T- 4514
CDC Rattan ----G-T- 4327
CDC Candle ----G-T- 4328
SB94912 ----G-T- 4328
Mochimugi-D ----G-T- 4442


4760 4770 4780 4790 4800 4810 4820 4830 4840 4850 4860 4870 4880 4890
Vogelsanger Gold CACCTTCGGTCGCAGGAACAGAGGTGTTCAAGGCAGTCCGAACTGCTCGCCGCCTGCAGTGGAACCACCACCATAAAGTTCGCCATTTT-GCA-TGAAGGAATACTCATATATGTGTAAGTTTCAAAATGGCGGTGCAAC 4891
Morex 4889
Shikoku Hakada #84 5000
CDC McGwire 4654
CDC Fibar 4653
CDC Alamo 4653
SB94893 4653
SH99250 4671
SH99073 4654
CDC Rattan 4467
CDC Candle 4468
SB94912 4468
Mochimugi-D 4582
4900 4910 4920 4930 4940 4950 4960 4970 4980 4990 5000 5010 5020
Vogelsanger Gold GACCGCCAAGATCGATGCAACATAACAAAATGAAAAAAGGGGAGCGATCATCTCCCGTGATGAGGATAGGGACAGCCACCTGTGACCATAGGCGACAACG-TGATCTGGATGATATGAACAAAGATGGTGGGAGG----- 5025
Morex TGGGA 5029
Shikoku Hakada #84 TGGGA 5140
CDC McGwire TG 4791
CDC Fibar TG 4790
CDC Alamo TG 4790
SB94893 TG 4790
SH99250 TG 4808
SH99073 TG 4791
CDC Rattan TG 4604
CDC Candle TG 4605
SB94912 TG 4605
Mochimugi-D TGGGA 4722
5030 5040 5050 5060 5070 5080 5090 5100 5110 5120 5130 5140 5150
Vogelsanger Gold --CACGGGAGATTGTCCTAAAAACTTTACTATTTCCATGGCTTGAAATCAACAATATTTCTTACTCAGTTTGCTGTTGAACTGTGAGTCTGCGAGGCACTAAACTTACTTAATAAGTTGTGCAACAACTC 5153
Morex GG C 5159
Shikoku Hakada #84 GG 5190
CDC McGwire 4791
CDC Fibar 4790
CDC Alamo 4790
SB94893 4790
SH99250 4808
SH99073 4791
CDC Rattan 4604
CDC Candle 4605
SB94912 4605
Mochimugi-D GG 4772

4.2. Alignment of the amino acid residues obatained from respective genomic sequences. . Sequences were aligned using the clustalW method of the Megaalign program (DNASTAR). Identical nucleotides were indicated by (•) whereas nucleotides absent is denoted as (─).

```
Vogelsanger Gold   MAALATSQLATSGTVLGVTDR-----FRRPGFQGLRPRNPADAALGMRTIGASAAPKQSRKAHRGSRRCLSVVVSATGSGMNLVFVGAEMAPWSKTGGLG   95
Morex              ......................-----..................................................R.....................   95
Shikoku Hakada #84 ......................SAPSM..HA........K...GTF....V..............N........R.....................  100
CDC McGwire        ......................-----..................................................................   95
CDC Fibar          ......................-----..................................................................   95
CDC Alamo          ......................-----..................................................................   95
SB94893            ......................-----..................................................................   95
SH99250            ......................-----..................................................................   95
SH99073            ......................-----..................................................................   95
CDC Rattan         ......................SAPSM..HA........K...GTF....V..............N........R.....................  100
CDC Candle         ......................SAPSM..HA........K...GTF....V..............N........R.....................  100
SB94912            ......................SAPSM..HA........K...GTF....V..............N........R.....................  100

Vogelsanger Gold   DVLGGLPPAMAANGHRVMVVSPRYDQYKDAWDTSVISEIKVADEYERVRFFHCYKRGVDRVFIDHPWFLEKVRGKTKEKIYGPDAGTDYEDNQQRFSLLC   195
Morex              ..................................................................................................   195
Shikoku Hakada #84 ..................................................................................................   200
CDC McGwire        ..................................................................................................   195
CDC Fibar          ..................................................................................................   195
CDC Alamo          ..................................................................................................   195
SB94893            ..................................................................................................   195
SH99250            ..................................................................................................   195
SH99073            ..................................................................................................   195
CDC Rattan         ..................................................................................................   200
CDC Candle         ..................................................................................................   200
SB94912            ..................................................................................................   200
Vogelsanger Gold   QAALEAPRILNLNNNPYFSGPYGEDVVFVCNDWHTGLLACYLKSNYQSNGIYRTAKVAFCIHNISYQGRFSFDDFAQLNLPDRFKSSFDFIDGYDKPVEG   295
Morex              ..................................................................................................   295
Shikoku Hakada #84 ..................................................................................................   300
CDC McGwire        ..................................................................................................   295
CDC Fibar          ..................................................................................................   295
CDC Alamo          ..................................................................................................   295
SB94893            ..........................................................T.......................................   295
SH99250            ..................................................................................................   295
SH99073            ..................................................................................................   295
CDC Rattan         ..................................................................................................   300
CDC Candle         ..................................................................................................   300
SB94912            ..................................................................................................   300

Vogelsanger Gold   RKINWMKAGILQADKVLTVSPYYAEELISGEARGCELDNIMRLTGITGIVNGMDVSEWDPTKDKFLAVNYDITTALEAKALNKEALQAEVGLPVDRKVPL   395
Morex              ..................................................................................................   395
Shikoku Hakada #84 ..................................................................................................   400
CDC McGwire        ..................................................................................................   395
CDC Fibar          ..................................................................................................   395
CDC Alamo          ..................................................................................................   395
SB94893            ..................................................................................................   395
SH99250            ..................................................................................................   395
SH99073            ..................................................................................................   395
CDC Rattan         ..................................................................................................   400
CDC Candle         ..................................................................................................   400
SB94912            ...........H......................................................................................   400
Vogelsanger Gold   VAFIGRLEEQKGPDVMIAAIPEILKEEDVQIILLGTGKKKFEKLLKSMEEKFPGKVRAVVRFNAPLAHQMMAGADLLAVTSRFEPCGLIQLQGMRYGTPC   495
```

```
Morex              ..................................................................................................  495
Shikoku Hakada #84 ..................................................................................................  500
CDC McGwire        ..................................................................................................  495
CDC Fibar          ..................................................................................................  495
CDC Alamo          ..................................................................................................  495
SB94893            ..................................................................................................  495
SH99250            ..................................................................................................  495
SH99073            ..................................................................................................  495
CDC Rattan         ..................................................................................................  500
CDC Candle         ..................................................................................................  500
SB94912            ..................................................................................................  500

Vogelsanger Gold   VCASTGGLVDTIVEGKTGFHMGRLSVDCNVVEPADVKKVATTLKRAVKVVGTPAYQEMVKNCMIQDLSWKGPAKNWEDVLLELGVEGSEPGIVGEEIAPL  595
Morex              ..................................................................................................  595
Shikoku Hakada #84 ..................................................................................................  600
CDC McGwire       ..................................................................................................  595
CDC Fibar          ................W.................................................................................  595
CDC Alamo         ..................................................................................................  595
SB94893           ..................................................................................................  595
SH99250           ..................................................................................................  595
SH99073           ..................................................................................................  595
CDC Rattan        ..................................................................................................  600
CDC Candle        ..................................................................................................  600
SB94912           ..................................................................................................  600
Vogelsanger Gold   AMENVAAP        603
Morex              ........        603
Shikoku Hakada #84 ........        608
CDC McGwire        ........        603
CDC Fibar          ........        603
CDC Alamo          ........        603
SB94893            ........        603
SH99250            ........        603
SH99073            ........        603
CDC Rattan         ........        608
CDC Candle         ........        608
SB94912            ........        608
```

Appendix 5.1. *SbeIIb* contig 1 covering exons 1 to 11 of CDC McGwire, SH99250 and SB94912. The complete CDC McGwire sequence is shown and regions showing polymorphism for SH94912 and SB99250 sequences are aligned below. Approximate transcriptional start (+1) is indicated and coding sequences are underlined with solid line. Retroelement within intron 2 is underlined with dashed line and regulatory element *Bbl* is shadowed.

```
CDC McGwire    GTTGATTGGTACCGACGCTTTGGAATCTTGAAGCTTGGTGGAAGATGTTCGGTGGACGCAGCGATCGCGAGGCTTCTTAGTTTTTGTCAATTCGTCATTGTCAGCATTTA   110

CDC McGwire    TTTCTTTTGTACTTCATGTGTTGTTTTTTTTAGGCGTTGCTGTTTTCTTCACCACCTATCGTTAAATGTATTGCTTTGTAATAAAAAAGGGGAAACCCTTTTTCAACAAG   220

CDC McGwire    GCGACGCCGTCCTGCAGCTACGCCACCGCAAAACCCCAAACCTCTTCGCCCCCATGCTACCATCCATGCAGCCGTCCGCCCGCGCGCGCGCGTTGGCCACGCCACCCGGT   330

                                                                                                          TATA box

CDC McGwire    GGTGGCCAGGCCACCACGCCGCCACTCTCGCGTGAAGGCTCCGTCCGCTTCCTCCTAGTCCCACTCTCTGCCCGTGCTATATAGCATCCGCCCTCCGCCCCCTACCAATC   440

               +1                                                                                         Exon 1

CDC McGwire    TTAGCACACCCCCCTGCGCCTCCTCATTTCGCTCGCGTGGGTTTAAGCAGGAGACGAGGCGGGGTCAGTTGGGCGGTTGGGTTGGATCCGATCCGGCTGCGGCGGCAGCG   550

CDC McGwire    ACGAG**ATG**GCGGCGCCGGCGTTCGCAGTTTCCGCGGCGGGGATCGCCCGGCCATCGGCTCGTCGATCCAGCGGGGCAGAGCCGAGATCGCTGCTCTTCGGCCGCAACAAG   660

CDC McGwire    GGCACCCGTTTCCCCCGTAATTGTTCGCGCCACCTTGCTCACTCGCATTCTCTCGTTCATTCGTATCGGGCTGCGGTATTCAGCGATCTTACGCTCCCTCTTGGTGTGGT   770

                                                                                                          Exon 2

CDC McGwire    GATGTCTGTAGGTGCCGTCGGCGTCGGAGGTTCTGGGTGGCGCGTGGTCATGCGCGCGGGCGGCCCGTCCGGGGAGGTGATGATCCCTGACGGCGGTAGTGGCGGAAGCG   880

CDC McGwire    GAACACCGCCTTCCATCGAGGGTTCCGTTCAGTTCGAGTCTGATGATCTGGAGGTACTTGACATATACAGTACTCCGTATTACCCTGAGTGAAATAGAACTATTATTGCC   990

CDC McGwire    ACCGCATTTTTACGGTTCACTCGAAATACCTGTTAACTTGCTATGGTTTTTGCTTTCATTGAGACGTCGACTAAATTCACTGAATTCCTATAATTTGGTAGACACCAAAA  1100

CDC McGwire    TATGTGCCTTTTAGATCAAAATATTCCGGTAGTTTCACTCGTATACAAGAAACAAACACACAACAACAAAGCCTTTAGTCCCAAACAAGTTGGGGTAGGCTAAAGGTGAA  1210

CDC McGwire    ACTCATAAGATCTCGCGATCGATTCATGGTTTTGGCACATGGATAGCAAGCTTCCACGCACCCCTGTTCATGGCTAGTTCTTTGGTGATGCTCCAATCCTTTAGAT--CT  1318

SB94912        ..............................................................................................................TT..  1320

SH99250        ..............................................................................................................--..  1318
```

Appendix 5.1. cont.

```
CDC McGwire    CTTTTTATGGACTCTTTTCATATCAAGTTCGGTCTACCCCGACCTTTCTTGACATTGTCAGCATGCTTTACCCGTCCGCTATGCACCACGGCTTCTCGAGGCCTACGCTG   1428
SB94912        ..........................................C.....................................................................   1430
SH99250        ..............................................................................................................   1428

CDC McGwire    AATATGCCCAAACCATCTTAGACGATGTTGGACAAGCTTCTCTTTAATTGGCGCTACCCCAACTCTATCTCGTATATCATCATTTCGAATTTGGTCTTTCTTTGTGTGGC   1538
CDC McGwire    CACACATCCATCTCAACATGCGTATCTCCGCCACACCTAGCTGTTGCACATGTCGCCTTTTAGTCGGCCAACACTCAGCGCCATACAACATTGCGGGTCGAACCATCGTC   1648

CDC McGwire    CTATAGAACTTGCCTTTTAGTTTTTGTAGCACCCTCTTATCACAGAGAATGCCAGAAGCTTGGCGTCACTTCATCC--ATCCGGCTTTAATTC--GATGGTTCACATCTT   1754
SB94912        ..............................................................................CC..............TT..............   1760
SH99250        ..............................................................................--..............--..............   1754

CDC McGwire    CATCGATATCCCCATCCTT-CTGCAACATTGACCCC-AAATAACGAAAGGTGTCCTTTTGAGGTGCCACCTGCCCATCAAGGCTAACACCCCCCCTCCTCCTCATGCTTA   1862
SB94912        ...................T.................C............................A.............................-..............   1869
SH99250        ...................-.................-..............................................................................   1862

CDC McGwire    GTAGTACTGAAATCGCACCTCATGTACTCGGTCTTAGTCCTACTAAGTCTAAAACCTTTCGATTTCAAGGTTTG-CCTCCATAACTCCAACTTCCTATTGACCCCGT--C   1969
SB94912        ..............................................................................A.............................TT.   1979
SH99250        ..............................................................................-.............................--.   1969

CDC McGwire    CGACTATCATCGACTAGCACCACATTGTCCGCAAAGAGCATACACCATGGGATATCTCCTTGTATATCCCTTGTGACCTCATCCATCACCAAGGCAAAAAGATAAGGGCT   2079
CDC McGwire    CAAAGCCGATCCCTGATGCAGTCCTATCTTAATCGGAAAGTCATCAGTGTCGCCATCACTTGTTCGAACACTTGTCACAACATTATCGTACATGTCCTTGATGTGGGTAA   2189

                                                    Bbl element
CDC McGwire    CGTATTTTGATGGGACTTCGTGTTTCTCCAAGACTCACCACATAACATTTCGCGATATCTTATCATAGGCCTTCTTCAAGTCAATGAACACCATATGCAAGTCCTTTTTT   2299
```

Appendix 5.1. cont.

```
CDC McGwire   TGCTCCCCATATCTCTACATAAGTTGTCGTACCAAGAAAATGGTTCCATGGTCGACCTCCCAGGCATGAAACGAAACTGATTTTTGGTCATACTTGTCATTCTTTTTAAG   2409
SB94912       .........C.CA.....................................................................................   2419
SH99250       .....................................................................................................   2409

CDC McGwire   CGGTGCTCAATGACTCTCTCCCATAGCTTCATTGTATGGCTCTCCGCCTATGTTGTCTCAACATAGCTGATCCCAAACCCGGGTAAAGGAGCAGGGTTGTGATAGGCTTG   2519
CDC McGwire   GCGAGCCAACGTCAAACTCAGCCACTCTTATGAAGATGAAACCCAAAAGTCGTATACAAGAATTCAAATTAATTTAAAATAAATCTAAAAATTCAACTAATTGGTTGAAT   2629

CDC McGwire   TTCAAGTGAGCGTTGTGGTCCTTTGGCCGAAATGTGAACCGAAATCACTGAATTTCACTGAATTTTCCCCTCAAAGAAGAAATTTCACTGGATTTCAGTACTGGCTGAAA   2739
SB94912       .....................................................................................-----..........   2744
SH99250       .....................................................................................................   2739

CDC McGwire   TATTTCCAGAACTGGAATTCAAAGTCTGCTATCCGGCAAAATTATATACCATTCTACTAAATTTTTGCTTATTTCACACGTAGGTTGCACTACATCCTCTTTCTAATTTG   2849
CDC McGwire   TTGGGGAATGGGGATTATTATCTTCTTGGTACCTGCCTGCATGACAATTGAAATCTGAAACAACACCCCACATGCGAGGCCTACACACTAGGTTGGTTTGCAACTATTTG   2959
                                                                                                        Exon 3
CDC McGwire   TGCCACGGTTCTGAACTTTTTGTCTTGCATGTCTTGCTAGGTTCCATTCATCGACGATGAACCAAGCCTGCACGATGGAGGTGAAGATACTATTCGGTCTTCAGAGACAT   3069
CDC McGwire   ATCAGGTTACTGAAGAAATTGATGCTGAAGGCGTGAGCAGAATGGACAAAGAATCATCCACGGTGAAGAAAATACGCATTGTGCCACAACCCGGAAATGGACAGCAAATA   3179
CDC McGwire   TACGACATTGACCCAATGCTCCGAGACTTTAAGTACCATCTTGAGTATCGGTATGCTTCGCTTCTTTTGTGTGCACTTTGAAAACAATTTACAGTCTTTGATAAGATGTG   3289
CDC McGwire   AATGGCTGATTGATGTGTCACGAAACTCTTGAAGTTCATAGTCACTCTTGTGTGTTCATTGTTCTGAGGTAACATGAATTTAGCATTTACTGAGGTGGTAACCGAACAAA   3399
CDC McGwire   AATAGGACAGTTTGGTAAGAACTGCAATGTAAAATACCCATAGCCACCCACTGTAATTGGGTACACTAATTAATGTATTTCTTGATGGGTTCTATGTGTTTTGAATATCT   3509
CDC McGwire   ATGCCAATTGAACATTCATTTCCCCTGTGTTGCTTTTGTAAGGATGAACCGATATGCCCAGATCAAACTGTACTAGCAGTCTCACTATGCCTTAATGGATCAAAAACAGA   3619
                                                                                                    Exon 4
CDC McGwire   TACAGCCTATATAGGAGAATACGTTCAGACATTGATGAATACGATGGAGGCATGGATGTATTTTCCCGCGGCTACGAGAAGTTTGGATTTGTTCGCAGGTGAAATTTCCT   3729
```

Appendix 5.1. cont.

Exon 5

```
CDC McGwire    GACTAGATAAGTATGTCTCTACCTTTTCTTTGTATCTTATCTATATTCCTCTTCCCATGCAGCGCTGAAGGTATCACTTACCGAGAATGGGCTCCTGGAGCAGATGTATG    3839
```

Exon 6

```
CDC McGwire    TTCTTCTGACTGTCTGATTGTTTACCTAAGTATACTAGTTTTATCTTTCAACTACTCTTGAATAATTACTGCTTCGAATTGCAGTCTGCAGCATTAGTTGGCGACTTCAA    3949
CDC McGwire    CAATTGGGATCCAACTGCAGACCATATGAGCAAAGTATGCATGTAGTTTCACAAATATATCATGTTTTCTTTGTAGATTAGTTTTTTTTAGATCTGCCTATATATTTAAA    4059
CDC McGwire    TGTGGTTGAATATACAACTTATGTATTCCGGAGTTGAGCCGTAAATATAGTTGTAAATTTTTAGGAGTATCAAATTCACTAGACTCTTTTCTTCACTTGCCTGTTGCACG    4169

CDC McGwire    AGCCCATTACTAGATATCAATGTTGATGATGCTTTTGTTGTATGAGGTCGAAGTGACAACATGCATGGTACTCTTTTATATAAGTAATGTTGCACCTTTTTTTTTATGAT    4279
SB94912        .............................................T.....................................................................    4284
SH99250        ..............................................................................................................    4279

CDC McGwire    TTAAGCATGATTTACTGATTTTTGTTATTCTAAGACACTGGGCGGTTTTACATAATAATGGTATTGGAGTAGGCCGACTGCATACCTCCTGAACTGTAGCTCCATGTATA    4389
```

Exon 7

```
CDC McGwire    GATTTGCAAATGCTCATATTCAATGTAATTGTTTCAGAATGACTTGGGTATTTGGGAGATTTTTCTGCCAAACAATGCAGATGGTTCGCCGCCAATTCCTCATGGCTCAC    4499
```

Exon 8

```
CDC McGwire    GGGTGAAGGTTTGTTTCTTCTCCTTGCCAACTGTGTTAGTCTCAGGAACATGTCCTGTATTACTCAGAAGCTCTTTTGAACATCTAGGTGCGGATGGATACTCCATCTGG    4609
CDC McGwire    GACAAAGGATTCAATTCCTGCTTGGATCAAGTACTCCGTGCAGACTCCAGGAGATATACCATACAATGGAATATATTATGACCCTCCTGAAGAGGTATTTTACATTTTTA    4719
CDC McGwire    CTTCATCTTTAGTGCTTTTAGATTTCAGAAATTTTTAATTGGAATGAAAATTATGATTTTTTTTCTCATGAAGCTTCACAAATGTTATTTCAAGCTGTCCTACTTCTTAT    4829
```

Exon 9

```
CDC McGwire    TTGCTGTTGGCATCTTAGGTTTGTATTCACTAACCAGTTATGAAATTCTTACATGCATATGCAGGAGAAGTATGTATTCAAGCATCCTCAACCTAAACGACCAAAATCAT    4939
CDC McGwire    TGCGGATATATGAAACACATGTTGGCATGAGTAGCCCGGTATTTCATCTTTACCATGTATTCCATAAATGATGTTAGCCATATGCAGTTCAAGTTAATTTACAGTTTGTT    5049
CDC McGwire    ACAATGATATTTTTGTGTTGTTGCGTTTCTTCTGTTTTATAAGTGAAAAATTATCGTTTTTTTGCTATGCCACTTGGTTAATACAATCTGAAAAATATAATTTGTGGACA    5159
```

Appendix 5.1. cont.

```
CDC McGwire    ATCAAGAATTAGATAATACGAATCTGAAAAAAATTTGCTGGAATAGTGTGATTTCAGTCAAATATGATGTTCTCAATGCTCGAGAGAAGTGTTAGATTGTGTAGCATCAA    5269

CDC McGwire    AAGCTGGCGTCCAGTTGTTCAAACGCTTAACTTGATGTAACTGTGAATGTTACATCTTTTGCTAGTATAAGTTCATATTTTTATCACTGTATTACATGTTTGATGTTTCA    5379

CDC McGwire    TCTCTGACAAAGGCCCAGACTAGAGCGCATCATGGCTTCTATTTCTCTAAACTCATTGTTATTAATCCACCATGAGCTTGAGAGCGGGTGTTAGAGTACTATATATATAG    5489

CDC McGwire    CCATGTAACCTTTCGTATTTACCCTATTGTATAAGGTGTTTCCTGCATATTCTATCCTGTACATGCATATATACTGGCCTTTGGCCTCTTAGAAATACAAGTTGCATATT    5599

CDC McGwire    CCTATCATCATCAACAATCTAATTAACCTCATTCCTTACAAACATTTGTAATTACATTTATTTTCCATGTCTCACTTCAGAAATAGAACAATAGAAATCATATAATCAAC    5709

CDC McGwire    GATCTAGATAATTCGGCGTATGCGTATGAATGTGGTGGCTTGCTATGCTCTTGTTCTAAAATATAAAAATAACTATGCTTGTTGGTAGCCTTTTTACATTAACACATGGG    5819

                                                                                                                    Exon 10

CDC McGwire    TAATTACTTGTTTCTTTGTGCTACCAGGAACCAAAGATCAACACATATGCAAACTTCAGAGATGAGGTGCTTCCAAGAATTAAAAGACTTGGATACAATGCAGTTCAAAT    5929
0
CDC McGwire    AATGGCAATCCAAGAGCATTCATACTATGGAAGCTTTGGGTAGTTCTCTGGGTCGATTTCTGGTTCTTTTACTTATCTATTTTGCCAATAGAACATATTTCAACTTTAGC    6039

CDC McGwire    AACTATTATATCAACTTTTCAGCTATTGTCTTCCTTTATTTTGTTTATGTGAGAGACTACTGCTTCGTGCTACTCCATGTGTTCTCAACTTCTCATTCAGAGTAGCCATC    6149

CDC McGwire    TATGAGTGGCCAACTCTATGTTGGTACTCTGGAAGTATCACCGGTTGGTTTGGTCTACAATAACATACTTCGTGTGATAGCCACCTAACAATACGATTACACACATAGTG    6259

CDC McGwire    CGGTAGTATGTTCCTCACCATACTAGCATAATGATTTGTTCTTTGTAGGAGTATATCATATTAGCTTCACTTCCAATGACATGGAAGCTGAAATAGCATTCAAATCATTT    6369

                                                                                                                    Exon 11

CDC McGwire    TTGTATTTTAAGTTCTGCCTTTTTCCTGTTTGATGATTAATACAACACCACTGTTATGTGTTTTTACTTCTATTTCAGGTACCATGTTACCAATTTCTTTGCACCAAGTA    6479

CDC McGwire    GCCGTTTTGGGTCCCCAGAAGATTTAAAATCCTTGATTGATAGAGCTCACGAGCTTGGTTTGCTTGTCCTGATG                                        6553
SB94912        ........................................................................                                        6558
SH99250        ........................................................................                                        6553
```

Appendix 5.2. Contig 2 sequences covering carrying exons 12 to 16 of CDC McGwire, SB94912 and SH99250 *SbeIIb* sequences. Underlined sequences indicate coding sequences.

Exon 12

CTTGGACGGTTTGAATGGTTTTGATGGCACGGATACACATTACTTTCATGGCGGCTCACGGGGCCATCACTGGATGTGGGATTCTCGTGTGTTCAACTACGGGAATAAGGAAGTATGGAA 120

Exon 13

CTATAGAACTCCTATATTGTCATCTGTTATGCATTTATCTGTTAATTAATTTTCCAACTGATTCCAACATTGTTATCTTTGTACAGGTTATAAGGTTTCTACTTTCCAATGCAAGATGGT 240

GGCTAGAGGAATATAAGTTCGATGGTTTCCGATTCGACGGCGCGACCTCCATGATGTATACCCACCATGGATTACAAGTAATGCATTGCTTGATTGTCCTTATTCTATCTTGACTACCTG 360

GGCAACTTTGATAGGATTACACCTAGCTAATATTTCCTTATATGTTGTAATATCAATTTTTATCCGAGCTTGAAACTTAATTTACTCTCTTTTTTCTGCATTATTGCACTCTATTTTAGG 480

Exon 14

TAACCTTTACAGGGAGCTACCATGAATATTTTGGCTTTGCCACGGATGTAGATGCAGTTGTTTACTTGATGCTGGTGAATGATCTAATTCACGCGCTTTATCCTGAAGCCGTTACTATTG 600

GTGAAGATGTAAGTGCTTCTGTATCTTTCTTGTAGTTTAGGTTATTCTGTCCATTCTTACAGGAGGTGCATACAGCAGTTGCTTTAGCTTTTGAAATGCAGTGCACACTGTGCCATTACT 720

TTGCAGCTATATATCGAGTTGAGAGCCATGGGAATCGTGTCATGCATGTGCATTGGCGTATCAAAAGCGTTATTCTTTGCCATTCTTATGGTCAAACTCTTACCGAAACATGCTTATCTG 840

ATCCTGCCTGTGCATAAATGCAATGCCACTATATTCATAGCTTAATATCAGGTACTAACATACTAACATAGATAAGATAGCTAGCTTAGATATTTGCATGTCAAGTTCCTAACAAAAGAA 960

CGCACCCCCCGCGTCGTTAGCGGAGCCCTCACCCCTGCCGCCATAGGGAGCATCCACCCTTCTCCTCTCCATTCACCACCGTCGGAGGGCAAAGCCCACGCGGAGTTGGCTCTCTTAAAG 1080

CATCTCCTATAGGTGCCCTATAATAAGGCACCTTTTGGTGGTTCGAGCAAATTTTGCAACACCTAAAACTGTTTTTTTTTCTACAACACCAAAGACCTTTAACTCCAATAGGTGCCCTAA 1200

AATAGGACACTAGTGTGACCGAAAATGCAGGAATTATTTTCTCTGCTCCTGGGTCATGGAAGAGTTTCTTATATATTGGGTCCAAACTCTCCTCCACTCCTGTGGTTAGACTAAATTTCC 1320

AACCATTTCACGAAACCACTAATTGGTCTTTCAGATTATGCAGGAATTATTAATTATATAATATGGCCTAAGGCCCATCTAACGTTCAACAATAATAACTTTGTTCAACTGAGGTGTTGG 1440

Exon 15

TTTTTCATTTGAATTCTCAGGTTAGTGGAATGCCTACATTTGCCCTTCCTGTTCAAGTTGGTGGGGTTGGTTTTGACTATCGCTTACATATGGCCGTTGCCGATAAATGGATTGAACTTC 1560

TCAAGTAAGCTTTCAAAATTGGTATGCATATGTTATTATTTTACTGGGTAGAAGATCTGATTGTGCAGTGTATATTAATGCAAATTAAATGTCCCTTTGCGTATGCAAATTAAATGTCCC 1680

TTTGCGTAACACTATTGCACATATGGACTTCAACATGAATGTCCACAAACATGTACCATTGTTAGTGTATTTGATATGGACACAATGTGATTAGTAGTTGCGACTTCGTAGTTTATAGAG 1800

TATAACAACAAAAGTAGGATACATGTACCCAACTTTTAGAAATTCTCATAATGATATTACACTTCTTTTAATATGTTATATCCTCACTTGCATCTCTCATCATTTTTATTGTCTCAGTTG 1920

TTTCAAGTGTCTGTTTTGGAACTGTAAAAGTTTGGTCTCGTGTTCTGGTTATTGATGTGGAGTATCATGTATCTGAAACATGAATTGCAACTTTTTATTCTAAACAGAGGAAGCGATGAA 2040

Exon 16

GGTTGGGAGATGGGTAATATTGTGCACACACTAACAAATAGAAGGTGGTTGGAAAAGTGTGT 2102

Appendix 5.3. *SbeIIb* contig 3 covering exons 17 to 22 of CDC McGwire, SH99250 and SB94912. Coding sequence is underlined. The complete CDC McGwire sequence is shown with regions showing polymorphism for contig 3 of SH94912 and SB99250 aligned below.

Exon 17

```
CDC McGwire    GAACGGACCTTCGACACCTAATATTGATCGCGGAATAGCACTGCATAAAATGATTAGACTTATCACAATGGCTTTAGGAGGAGAGGGTTATCTTAACTTTATGGGAAATG   110
CDC McGwire    AGTTCGGGCATCCTGGTGAGATTAAATATTTTCTTGCATGTTATTTGAATCTTGACCAAACATTTGATTTGAATCTTGACATGGTAGTTCTAATGTCTGAGGAAGGCATT   220
```

Exon 18

```
CDC McGwire    ATTGTAAGGTTTTTATTGAAACATTTATTGTGTAGAATGGATAGACTTTCCAAGAGGCCCACAAGTACTTCCAACTGGTAAGTTCATCCCAGGAAATAACAACAGTTACG   330
SB94912        ...T.............T...........T.....................................................................................   330
SH99250        ...........G..G.............T.....................................................................................   330

CDC McGwire    ACAAATGCCGTCGAAGATTTGACCTGGTAAGTTTTCTTTCGAGTGTTGCTAACAGCAATAATTTTACTACCTCCATTGAGTCAATTTAGTTTTGGTTAAAAAAATATTCA   440
CDC McGwire    ATTTAGAGCTTCTTTTACCTCTTCTAGTGCTTTATTGAACAAGGTACCATATATGACGTTCATCTTGGTTCTGTGCTCTTATTCTCATTAGCATTTGGGTAGGACTATAA   550
CDC McGwire    CTTATTTTATTTTTTTGAGAAAACACAAAAGCTTTACATCTGGATGTATTGATTGAAGAAGTAATATGTCAAAGCTTAGCCATACTTTTGACCGAGGCATCGATAAGAAA   660
```

Exon 19

```
CDC McGwire    AACTTTCTTGTTTTACTTGTCAGGGTGATGCAGAATTTCTCAGGTATCATGGTATGCAGCAATTTGATCAGGCAATGCAGCATCTTGAGGAAAAATATGGCGTATGTACT   770
SB94912        ....C...C.....................................................................................................   770
SH99250        ...AAA....AAACTT..............................................................................................   770

CDC McGwire    ATTTACATTATTTTCTTTTCCTATGAAATTGAGTCGCCATCACTTGGTGTTTTACCCACTAAAAACATCACACTAGGAGTTGGTCTTTCATTCTTTCTGTCATGCCATTC   880
CDC McGwire    TATTCATATTCACTTAACTGTCACATTAGGAGGAAGTTTGGTATGATGGTACAAGGAACATCCACTAAATCTGATTAACTAATCCGCATAACAAAAATGCAGCATTTTGC   990
CDC McGwire    ATTCATGTACTCTCACTTTGTAATAAATCATTTTAATATGTTGTCCGTTTTAATTATTTAGCTTACAAAATAGCAAAACCTTATCGGTGTTTCGAAGTTGTCCTCCCCCT  1100
CDC McGwire    TTTGGGCTTGATGCTTGTATTCTTTTCTCTACATTATTGAAATAAGGAAGAGCACACGACCTTTCTTTTGAAATATAACCTTAACAGAACTAGTTACCTTGATAAATGCT  1210
CDC McGwire    GGATAGAAGAATTAAATTAGTACAGAAGGTAGTGGGATAATACTATAATCTTCCTTTGCTTCCTTATTAAGGGTCAGTCGTCCATGTTCTCCTTTATATTATCTCCATAC  1320
```

```
CDC McGwire    ATCAACAATGAAAGCATTAACCTCTTCAGGAGAAAGCTGCAGATTTTCTCCTCCTTTGAAAACACCTGATTTGTCTTCACTTTGTGAGTGCTAAACTTAGATTTGAAATC    1430

CDC McGwire    GGGGTAGTGGATTTACAGTACCTCTACTACTGCTTTCTTGCCTTAAAATTATATCTGGTAATGTAGGCAGATATTAGTAAGTTCATTATGTGGCACCTATGAATATTCGC    1540

                                                                                                                    Exon
CDC McGwire    TGATGAAACAGGCCATGTAA--TTGCAGTTTATGACATCAGACCACCAGTACGTATCTCGGAAACACGAGGAAGATAAGGTGATCGTGTTTGAAAAAGGGGACTTGGTAT    1648
SB94912        ..........CC...CCCC.--.....................................................................................    1648
SH99250        ............GG...GGGAT.....................................................................................    1650

CDC McGwire    TTGTGTTCAACTTCCACTGGAGTAATAGCTATTTCGACTACCGGGTCGGTTGCTTAAAGCCTGGGAAGTACAAGGTATTCAGCTATTGCCATTCCTCATGTTAAGATTGT    1758

CDC McGwire    TAGTTTTCTTTGCTGTACACTTGTTCTATACACATTATCTCATGCTCATGCTTGAATACACTGATAGTTAGGTGTGAGGGAGTATTGGTAGTCTGACTAGGTTCTGTTTC    1868

CDC McGwire    CTTCTTAATTTTAGAAAGAAAAAAAAAACTTTTTGGGGTTGCTTTTGTAGTACGAGCAAACCTAGTAAGCAAAATCCAAATGATTTATACATGTTGGAATTAATTTCTCT    1978

CDC McGwire    CACACAAATCTTTTAAGTTTAATCAAAATCCAAATGATTTATACAATGCCTATGCTATGTTCTGGCTTTCAAAATTTATTCAAAAATTGTTGCTGCCCGACTTCCCTGTC    2088

CDC McGwire    ACACGTGAGAAGGGGTGTTAAAGTGTATATGTGGATTGCCTAACCCTTTCCATTAGCTTGGACTTTTAGTTGCACTGTGTAGTGCATGAAGCCTAACATGATATGAGGAC    2198

CDC McGwire    CTAAGGTCTTGAGTTCAAGTTCTGGCTTTCAAAATTTATTCAAAAATTGCTGCTACCCGACTTCCCCGTCACACGTGAAAGGGGTGTTGAAGTGTATAAGTGGATTGCCT    2308

CDC McGwire    AACCCTTTTCATCAGTTTGGACTTTTGATTGCATTGGCTACTGCATGAAACTTAACAGATAGTTGTATTCCATCTTTACTTGTAGATGACTTTGACTATTATATGATGAA    2418

CDC McGwire    TAAAGTTCTTTACCTTAAAAAAAGAATTGACCACCTATGTTGGTACCTATGCCGGGTAGCTATATGCCTAATCTTTTGATCCTCCCATTGTTCAACCTGGTATTCTTTTC    2528

CDC McGwire    GAGCAACCAATAGATGCTCTGCTTTTTCATTAATAAGAATAAGAATTGACTCGTTAATAAGGAAAACGGCTGAAAACCAATACATCATCATCGCACACAACGTCCTTGAG    2638

CDC McGwire    GCCCTGCACCCGACGAGCCGCCAGCTCCACCACTCGAGCAACTGGAGCCGACACCCTAACACCAGATCCGAAGCCATCCACGACAGCTCCAAGACGGTGCACCATACAAG    2748

CDC McGwire    TCGACGAATCAGTCACTCAAGCAACTAGAGACGACACCCTATAATACCATGACGGTTTTCTAAGTCGCCGCTCCGACTAACAAAAGAAAGAACAAACACGCACGTCGATC    2858

CDC McGwire    TCGAGTCTGCGCATCAAGCGAGTTGCAGGCTCCGGAACCCAAGCAACCGGAGCAGACACCCTGCAACCATTATCGAAGCCGGGTCGAGAACGCAAAGTTTCTATTCTGAG    2968

CDC McGwire    CATATTGTTCAGTCTACTCATGTTTTGGAATTTACCTAGAAAGCTTACTCAAAATTCCTAGAATTAGTTCTGATTTCTGAAGCAGGTACGTATGTACTCCCTCCGTCTCA    3078

CDC McGwire    TAATATAAGACGTTTTTAACACTACACACTACTGCGGTAAAAAACATCTTATATTAAGGGACTGAGGGAGAACCTTCTTGCGTTGGGTGCTGAAAGTATATTCTTTCTAC    3188
```

Exon 21

```
CDC McGwire    CAGGTGGTGTTAGACTCAGACGCTGGACTCTTTGGTGGATTTGGTAGGATCCATCACACTGGAGAGCACTTCACTAATGTAAGCCTTTTGCTCAGACGTGGTTGCTTTTA   3298
SB94912        ..............................................................................C.C....TCG..............   3298
SH99250        ..............................................................................T.TT.................   3300
```

Exon 22

```
CDC McGwire    GTATATGAGTACTTGTGTAATCTCATTTCTGGTAAACCTGAAATAACATATGCTTCACATGCACCTTGCAGGGCTGCCAACATGACAACAGGCCCCATTCGTTCTCAGTG   3408
CDC McGwire    TACACTCCTAGCAGAACCTGTGTTGTCTATGCTCCAATGAACTAACAGCAAAGTGCAGCATGCGCATGCGCGCTGTTGTTGCTTAGTAGCAACATAAATCGTATGGTCAA   3518
CDC McGwire    TACAACCAGGTGCAAGGTTTAATAAGGTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTGCTTCAACCAGTCCTGGATAGACAAGACAACATGATGTTGTGC   3628
CDC McGwire    TGTGTGCTCCCAATCCCCAGGGCGTTGTGAGGAAAACATGCTCATCTGTGTTACCATTTTATGAATCAGCAACGATACTTCTCCCA                              3714
```